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Metabolism and Function of Choline and Sphingolipids in Rat Sympathetic Neurons

by

Miguel Bussière

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

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Abstract

Cholinergic neurons utilize choline for synthesis of membrane phosphatidylcholine (PC) and for synthesis of the neurotransmitter acetylcholine (Ach). Studies in the first half of this thesis examined the mechanisms regulating the distribution of choline between PC and Ach synthesis in rat sympathetic neurons induced to become cholinergic by retinoic acid treatment. Upon cholinergic differentiation, choline acetyltransferase activity was increased resulting in greater Ach synthesis, and the activity of choline kinase, which synthesizes phosphocholine, the first intermediate in PC synthesis, was decreased. Overall PC synthesis was unaffected by the decrease in choline kinase activity. Ach is thought to be synthesized predominantly within axon terminals from extracellular choline taken up by a specialized choline transporter. [^{3}H]Choline supplied to isolated distal axons was incorporated into both Ach and PC within distal axons. [^{3}H]Choline taken up by cell bodies, however, was incorporated solely into PC within cell bodies and distal axons. These results suggest that choline derived from cell bodies is not utilized for Ach synthesis and are consistent with the view that choline destined for Ach synthesis is taken up via a specialized choline transporter localized to axon terminals.

In the second part of this thesis, the role of glycosphingolipids (GSLs) in axonal growth was examined. The effect of two inhibitors of sphingolipid synthesis, fumonisin B_1 and D,L-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), on axonal growth was determined. Although both fumonisin and PPMP inhibited GSL synthesis, PPMP, but not fumonisin, induced an accumulation of [^{3}H]palmitate-labeled ceramide and impaired axonal growth. Moreover, cell-permeant C_6-ceramide mimicked the effect of PPMP. Thus, newly synthesized GSLs are not essential for axonal growth, while the bioactive lipid ceramide acts in distal axons, but not cell

bodies, as a negative regulator of axonal growth. A distinct localization of acid sphingomyelinase activity to cell bodies and enrichment of neutral sphingomyelinase activity in distal axons was observed. Since axonal transport of ceramide is inefficient, an elevation of ceramide within distal axons might be produced by activation of neutral sphingomyelinase. Ceramide attenuates NGF-activated protein tyrosine phosphorylation in distal axons and therefore this might be a mechanism by which ceramide inhibits axonal growth.

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Abbreviations

Acetyl-CoA	acetyl-coenzyme A
Ach	acetylcholine
AchE	acetylcholinesterase
ATP	adenosine triphosphate
CAPK	ceramide-activated protein kinase
CAPP	ceramide-activated protein phosphatase
CDP-choline	cytidine diphosphocholine
ChAT	choline acetyltransferase
CK	choline kinase
CoA	coenzyme A
CPT	choline phosphotransferase
CT	cytidine:phosphocholine cytidylyltransferase
CTP	cytidine diphosphocholine
DG	diacylglycerol
diC_8-glycerol	dioctanoylglycerol
EGF	epidermal growth factor
FB_1	fumonisin B_1
GPI	glycosylphosphatidylinositol
GSL	glycosphingolipid
HACT	high affinity choline transport, sodium-dependent and HC-3-sensitive

HC-3	hemicholinium-3
kDa	kilodalton
NBD-C_6-ceramide	*N*-[7-(4-nitrobenxo-2-oxa-1,3-diazole)aminocaproylsphingosine
NGF	nerve growth factor
$p75^{NTR}$	p75 neurotrophin receptor
PC	phosphatidylcholine
p-choline	phosphocholine
PDGF	platelet-derived growth factor
PDMP	D,L-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
PPMP	D,L-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol
RA	*all trans*-retinoic acid
SAPK	stress-activated protein kinase
SAPs	saposins or sphingolipid activator proteins
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM	sphingomyelin
SMase	sphingomyelinase
SPT	serine palmitoyltransferase
TNF-α	tumor necrosis factor- α
UDP-glucose	uridine diphosphate-glucose

CHAPTER 1

INTRODUCTION TO CHOLINE METABOLISM IN CHOLINERGIC NEURONS

1.1 INTRODUCTION TO THE NERVOUS SYSTEM

The nervous system is a complex array of specialized cells which functions to collect sensory information from the body, to interpret and store this information and, if necessary, to react to this information by initiating an appropriate motor response. The two major divisions of the nervous system are the central nervous system and the peripheral nervous system. The central nervous system consists of the brain and spinal cord. It is the control center for the entire nervous system. The central nervous system receives and processes sensory information and coordinates and controls motor output. The peripheral nervous system is composed of nerves emerging from the brain (cranial) and spinal cord (spinal). It is made up of sensory neurons that conduct sensory information from various parts of the body to the central nervous system and motor neurons which conduct impulses from the central nervous system to muscles and glands.

The nervous system is composed of two basic types of cells: neurons and neuroglia.

Neurons conduct electrical impulses or nerve signals from one part of the body to another. It is estimated that there are approximately 100 billion neurons in the human nervous system. Although neurons differ in their size, shape, connections with other cells, the type of neurotransmitters they synthesize and secrete, etc., all neurons share a common distinct morphology (Fig. 1.1). The structure of a typical neuron, described by Ramón y Cajal more than a century ago (1), can be subdivided into four regions:

the cell body, the dendrites, the axon and the axon terminal (Fig.1.1). The cell body of a neuron (5-135 μm in diameter) is generally considered to be the metabolic center of the neuron and contains a nucleus as well as typical organelles such as lysosomes, mitochondria, endoplasmic reticulum and Golgi apparatus. The dendrites are short, thick, highly branched cytoplasmic extensions of the cell body. Their function is to receive and transmit nerve impulses toward the cell body. The axon is usually a single long tubular extension of the cell body whose primary function is to conduct nerve impulses from the cell body to a distant target. Axons vary in length from as short as a few millimeters to as long as a meter. Many axons are surrounded by an insulating sheath called the myelin sheath. Although membrane and protein synthesis has long been thought to occur solely in the cell body of a neuron, more recent studies have found that membrane lipid biosynthesis also occurs within axons (2, 3) and that axonal synthesis of lipid plays a primary role in membrane biogenesis during axonal growth and regeneration (4). At its distal end an axon divides into many fine branches which have specialized endings called axon terminals. The axon terminals of a neuron lie in close proximity to the cell membrane of effector cells such as other neurons or muscle cells (Fig. 1.1). The contact site between an axon terminal and an effector cell is called the synapse. Transmission of a nerve impulse from a neuron to an effector cell often consists of the release of a chemical substance, known as a neurotransmitter, into the synapse that acts on receptors on the effector cell. The first neurotransmitter to be discovered (5), and probably the best characterized, is acetylcholine (Ach) which is released by many neurons in the central and peripheral

nervous systems and at neuromuscular junctions. Neurons that secrete Ach as their neurotransmitter are called cholinergic neurons.

Neuroglia play a supportive and protective role in the nervous system. Some of the many functions ascribed to neuroglia are: to provide support to neurons by forming fibers that hold neural tissue together, to help bind neurons to blood vessels and supporting structures, to engulf and destroy microbes and cellular debris and to synthesize and maintain the myelin sheath that covers the axons of many neurons. In the central nervous system, the glial cells that produce this myelin sheath are called oligodendrocytes, whereas in the peripheral nervous system, this function is performed by Schwann cells. The myelin sheath insulates the axon and allows fast nerve impulse conduction. The blood-brain barrier, a boundary between the circulation and the internal milieu of the brain, is formed in part by astrocytes, star-shaped glial cells with many cellullar processes which are associated with neurons and blood vessels. The blood-brain barrier helps to buffer the fluid environment of the brain from the large fluctuations in nutrient and metabolite levels which occur in the blood.

1.2 Choline Utilization in Growing Cholinergic Neurons

1.2.1 Biosynthesis of phosphatidylcholine and acetylcholine

In mammalian tissues choline is an essential precursor for the biosynthesis of phosphatidylcholine (PC), the major phospholipid component of cellular membranes

(Fig. 1.2). In the nervous system, cholinergic neurons use choline for an additional purpose: choline is acetylated to generate the neurotransmitter Ach (Fig. 1.2). Although research on the biosynthesis and function of these two end products of choline metabolism spans more than 50 years, the mechanisms that govern the distribution of choline between PC and Ach biosynthesis in growing cholinergic neurons have not been clearly elucidated.

It has been suggested that the unique ability of cholinergic neurons to use choline for Ach synthesis might contribute to their selective vulnerability in Alzheimer's disease and other cholinergic neurodegenerative disorders (6). Choline incorporated into membrane PC can be liberated by the action of phospholipase enzymes and utilized for Ach synthesis (7-10). Cholinergic neurodegeneration might be caused in part by the over-utilization of membrane PC as a source of choline for Ach synthesis which would lead to decreased membrane integrity and compromised neuronal cell function (6). Choline availibility has been shown to be critical for brain development. Periods in brain development that are most sensitive to choline deficiency or supplementation correlate with the neurogenesis of cholinergic cells and with synaptogenesis (11). Thus, understanding the regulation of choline utilization in neurons is an important goal for modern biomedical research.

1.2.2 Regulation of phosphatidylcholine biosynthesis

Two pathways exist for the biosynthesis of PC, the cytidine diphosphocholine (CDP-choline) pathway, also called the Kennedy pathway, and the phosphatidylethanolamine *N*-methyltransferase pathway. Phosphatidylethanolamine *N*-methyltransferase catalyzes the sequential methylation of phosphatidylethanolamine to generate PC (12). This pathway is most active within liver although low activity has been detected in other tissues (12). The contribution of this pathway to PC biosynthesis in neurons has been found to be quantitatively insignificant (3, 12, 13). The CDP-choline pathway discovered in 1956 by Kennedy and Weiss (14, 15) is the predominant pathway for the synthesis of PC in all mammalian tissues. Choline taken up by a cell is phosphorylated to phosphocholine and subsequently activated by its conversion to cytidine diphosphocholine (CDP-choline). The phosphocholine moiety of CDP-choline is then transferred to diacylglycerol leading to the formation of PC (Fig. 1.2).

Transport of Choline into Cells. Choline utilized for Ach and PC synthesis in the brain is derived from the diet, enters the circulation and traverses the blood-brain barrier into the intercellular fluid (16). The plasma choline concentration is in the range of 10-20 μM (17, 18) and the concentration in the intercellular fluid of the brain (assumed to be equivalent to the choline concentration in the cerebrospinal fluid) is approximately 5-10 μM (13). Choline is transported into non-cholinergic cells primarily via a facilitated diffusion mechanism which has a high capacity but low

affinity for choline (K_m>30 μM), is energy- and Na^+-independent and is relatively insensitive to inhibition by the choline analogue hemicholinium-3 (HC-3) (19). A high-affinity choline transport system (K_m<5 μM) is also present in some non-cholinergic cells which is temperature- and energy-dependent, may or may not be Na^+-dependent and is sensitive to inhibition by elevated concentrations of HC-3 (19). Both of these choline transport systems supply choline for the synthesis of membrane PC.

In cholinergic neurons, two choline transporters have also been reported to exist with low and high affinity for choline (20, 21) (Fig. 1.3). The low affinity carrier has similar characteristics to that found in non-cholinergic tissue and is thought to supply choline for PC synthesis (19, 22, 23). The cholinergic high affinity choline transport system (HACT) has several unique characteristics which have been elucidated primarily from studies examining choline transport in synaptosomal preparations. It is thought to be localized exclusively to cholinergic nerve terminals (24, 25) and its regional distribution in the brain corresponds with choline acetyltransferase activity and Ach concentration (21, 26). Cholinergic HACT is tightly linked to the synthesis of Ach (22, 23, 27), is temperature-, energy- and Na^+-dependent, sensitive to inhibition by low concentrations of HC-3 (22, 23, 27) and its capacity increases or decreases in parallel with changes in neuronal activity (28-31). The role of cholinergic HACT in regulating Ach synthesis will be discussed in greater detail in Section 1.2.3 below.

Isolation and biochemical characterization of choline transporters has proceeded slowly. Genes for choline transporters from yeast (32) and bacteria (33) have been cloned. The yeast choline transporter is a 62 kDa protein containing up to 8 transmembrane regions (34). This transporter is energy-dependent, has a high affinity for choline (K_m=0.56 μM) and is functionally linked to PC synthesis (35). The bacterial choline transporter has been shown to be regulated by and to play a role in the adaptation to osmotic stress. Under conditions of high osmolarity, bacteria take up choline and convert it to betaine as an adaptive response to overcome the osmotic strength of the environment (36, 37). Several groups have attempted to purify and clone the cholinergic HACT protein with limited success. Choline transport activity has been solubilized from brain and *Torpedo* electric organ, a rich source of cholinergic terminals, and reconstituted into liposomes (38, 39). This activity exhibited similar characteristics to the HACT activity of native membranes. Several groups have achieved the expression of choline transport activity from mRNA isolated from rat spinal cord and *Torpedo* electric lobe injected into *Xenopus* oocytes (40, 41). A high affinity choline transporter has recently been solubilized from locust synaptosomal membranes and reconstituted into liposomes (42). The protein has a molecular mass of 90 kDa which is reduced to 65 kDa upon treatment with endoglycosidase F, indicating extensive glycosylation of the protein. This transporter exhibited properties similar to the mammalian cholinergic HACT system in terms of its requirement for Na^+ and sensitivity to HC-3. A monoclonal antibody raised to the

locust choline transporter, however, did not cross react with mammalian tissue (43). Rylett *et al.* have identified two polypeptides of 35 and 58 kDa from *Torpedo* synaptic membranes (44) and rat striatal synaptosomal membranes (45) which bind [^{3}H]choline mustard aziridinium ion, an irreversible inhibitor and affinity ligand for cholinergic HACT. Association of [^{3}H]choline mustard aziridinium ion with the 35 and 58 kDa polypeptides required Na^+ and was blocked by HC-3. Either one or both of these polypeptides may represent the choline binding site of the cholinergic HACT system. Although the cloning of a mammalian cholinergic HACT was reported by Mayser *et al.* (46), it now appears that this protein was actually a creatine transporter (47). Further characterization of choline transport in neuronal as well as non-neuronal cells awaits the purification and cloning of a mammalian choline transporter.

Choline Kinase (CK) catalyzes the phosphorylation of choline, in the presence of ATP, to form phosphocholine (Fig. 1.2). This enzymatic reaction commits choline to the synthesis of PC via the CDP-choline pathway. The first demonstration of the existence of this enzyme was in 1953 by Wittenberg and Kornberg (48). Since then cytosolic CK has been purified to homogeneity from rat liver (49), brain (50), kidney (51) and monkey lung (52). The subunit molecular mass of the enzyme is reported to range from 44 to 47 kDa by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Native molecular mass measurements suggest that CK exists as a dimer in brain (50) and a tetramer in liver (49). Several isozymes of choline kinase are thought to exist on the basis of differences in electrophoretic mobility,

chromatographic behavior, isoelectric point, antigenicity and inducibility. CK cDNAs from human glioblastoma (53), rat liver (54) and yeast (55) have been cloned and encode proteins with relative molecular masses of 49.7, 52.1 and 66.3 kDa, respectively. The enzyme specifically requires adenosine triphosphate (ATP) as a phosphate donor and has an absolute requirement for Mg^{2+} ion. The reported K_m of the enzyme from rat liver for choline varies from 13 to 110 μM and 1.5 mM for Mg^{2+}-ATP (19, 49).

Although the second enzyme of the CDP-choline pathway, cytidine triphosphate:phosphocholine cytidylyltransferase (CT), is generally considered to catalyze the regulatory step for this pathway, a number of reports indicate that under certain conditions CK may also play a regulatory role. CK activity is increased by treatment with estrogen (56), mitogens (57, 58) and in essential fatty acid deficiency (59). Under these conditions, increased CK activity results in increased PC synthesis. As well, in the liver, certain isoforms of CK are selectively induced by treatment with the carcinogen 3-methylcholanthrene or the hepatotoxic compound carbon tetrachloride (60). Interestingly, in explants of superior cervical sympathetic ganglia, a correlation between the extracellular concentration of choline and the activity of CK was observed (61). CK enzyme activity was inhibited at low extracellular concentrations of choline (1-5 μM) but increased as the external concentration of choline was raised (10-50 μM). On the other hand, the activity of choline acetyltransferase (ChAT), which catalyzes the synthesis of Ach (see Section 1.2.3),

remained elevated at low concentrations of choline in the incubation medium but fell as the extracellular concentration of choline was raised (61). The observed opposite changes in the activities of these two enzymes in response to changes in external choline concentration suggests that CK and ChAT might regulate the distribution of choline between PC and Ach biosynthesis.

Cytidine triphosphate:phosphocholine cytidylyltransferase catalyzes the second reaction in the CDP-choline pathway, the synthesis of CDP-choline and pyrophosphate from phosphocholine and cytidine triphosphate (Fig. 1.2). Under most metabolic conditions, this is the rate limiting and regulated step of PC synthesis via the CDP-choline pathway (62, 63). The enzyme has been purified (64, 65) and a cDNA cloned from rat liver (66) which codes for a protein of molecular mass 41.7 kDa. Much research has been devoted to the elucidation of the mechanisms regulating CT. A brief summary of the key findings in this field follows but the reader is referred to excellent reviews for a more extensive discussion of this area (62, 63, 67). CT resides in two intracellular pools: a soluble pool and a membrane-bound pool (68). Numerous studies suggest that changes in the rate of PC synthesis are associated with redistribution of CT between the soluble (inactive) pool and the membrane-bound (active) pool (62, 63, 67). Several factors have been demonstrated to govern the distribution of CT and its activity. Addition of fatty acids to cells (69, 70) and elevation of intracellular diacylglycerol levels (71, 72) have both been shown to promote the translocation of CT to membranes and activation of the enzyme, resulting

in increased PC synthesis. A decrease in the concentration of PC in cellular membranes is also associated with a redistribution of CT to membranes and a concurrent increase in the synthesis of PC (73, 74). The phosphorylation state of CT, although previously believed to play a role in the subcellular distribution of CT (75, 76), appears not to play a role in CT translocation (77). The subcellular localization of CT has recently been reexamined. The soluble and membrane forms of CT have been generally believed to be cytosolic and bound to the endoplasmic reticulum, respectively (68, 78). Recent evidence suggests, however, that in some cells CT is predominantly present in the nucleus (79, 80). In agreement with these findings, nuclear localization sequences are present in the primary sequence of CT (81). Reexamination of this question by the Vance group has led to the conclusion that this nuclear localization of CT does not apply to all cell types (82). These findings are supported by the detection of CT activity extranuclearly in the axons of rat sympathetic neurons (2, 3). The significance of the presence of nuclear CT in some cells remains to be demonstrated.

Choline phosphotransferase catalyzes the final step in the *de novo* synthesis of PC, the condensation of CDP-choline and diacylglycerol (Fig. 1.2). In subcellular fractionation studies, this integral membrane protein was predominantly found associated with the endoplasmic reticulum (83, 84) but was also present in Golgi membrane fractions (78). Little information about choline phosphotransferase is available, relative to other enzymes, due to difficulties in the purification of this

enzyme. A partial purification has been achieved (85), however, and the enzyme has been cloned from yeast (86) by genetic complementation of a mutant defective in this enzyme. The yeast gene encodes a 407 amino acid protein with a predicted molecular mass of 46.3 kDa. Choline phosphotransferase is thought to be present in excess *in vivo* and is, therefore, not regulatory for the synthesis of PC (87). The rate of the reaction is controlled by the supply of CDP-choline and diacyglycerol (87, 88).

Substrate Channeling of the water soluble intermediates of the CDP-choline pathway has been reported by Spence and coworkers (89). Using electropermeablilized rat glioma cells, these researchers have provided evidence that the intermediates of this pathway are not freely diffusible in the cell, but are channeled towards PC synthesis. Furthermore, the integrity of this compartment was found to be dependent on intracellular Ca^{2+} levels (90, 91). Whether this compartmentation exists in other cells, in particular in cholinergic neurons, remains to be demonstrated.

1.2.3 Regulation of acetylcholine biosynthesis

Ach was the first neurotransmitter to be described more than 70 years ago (5). It is synthesized in cholinergic neurons from the substrates choline and acetyl-coenzyme A (acetyl-CoA) through a reaction catalyzed by the enzyme choline acetyltransferase (ChAT) (Fig. 1.2 and 1.3) discovered by Nachmansohn and Machado in 1943 (92). The amount of Ach stored within the terminals of cholinergic neurons is maintained

at a near constant level under a variety of physiological conditions. The rate of synthesis of Ach is able to adapt to changes in the release of Ach in order to maintain a stable intracellular level of the neurotransmitter (13, 93). These findings have driven investigations to determine the biochemical mechanisms regulating the synthesis of Ach. Two factors which play a role in the regulation of Ach synthesis are the supply of choline and modulation of ChAT activity.

HACT and choline supply. As early as 1961, it was speculated that a specialized choline transport system must be located in a membrane near the site of Ach synthesis (94) (Fig. 1.3). It was not until 10 years later that the importance of this transport system as a rate limiting and regulatory step in the synthesis of Ach started to be recognized (20, 21, 26, 95). As mentioned earlier (Section 1.2.2), kinetic studies demonstrated the presence of two choline transport systems with different affinities for choline, high (HACT) and low, in nervous tissue (20, 21, 26, 95). The low affinity choline transport system was neither dependent on Na^+, nor was it associated with the synthesis of Ach. The HACT system was found to be highly Na^+-dependent, was associated with the efficient conversion of choline to Ach and its distribution in the brain paralleled that of ChAT (20, 21, 26, 95 and see Section 1.2.2). The primary role of this transporter is thought to be the recycling of choline generated by the hydrolysis of Ach to choline and acetate by the enzyme acetylcholinesterase in the synaptic cleft (Fig. 1.3). In sympathetic ganglia, recycled choline accounts for 50-60% of the supply of choline for new Ach synthesis (93, 96), the remainder is recruited from the

extracellular fluid (96). It is estimated that 60-70% of the choline transported by HACT is acetylated (20, 21, 26, 95) and that acetylation is more efficient at lower extracellular choline concentrations (20, 21, 26, 95, 97). These observations have led to speculation that HACT and ChAT are physically (98) or kinetically coupled (99) such that choline transported via HACT is rapidly acetylated. The nature of this coupling, however, remains to be demonstrated. The hypothesis that HACT is a rate-limiting step of Ach synthesis is further supported by the observation that inhibitors of HACT, such as HC-3 and choline mustard aziridinium ion, inhibit Ach synthesis in proportion to their inhibition of HACT (100, 101).

One of the properties of HACT which implies a regulatory role of this transporter in Ach synthesis is that its capacity appears to be coupled with neuronal activity. Nerve stimulation induces the release of stored Ach from nerve terminals and concurrently accelerates Ach synthesis. Treatments which alter neuronal activity both *in vivo* and *in vitro* cause parallel changes in the rate of transport of HACT. Thus, anesthetics or drugs shown to decrease Ach turnover, decrease the rate of transport of HACT (28, 29). Conversely, treatments which increase cholinergic activity, such as convulsants or depolarization by exposure to high concentrations of K^+ ions, increase the rate of transport of HACT (28-31). The molecular events underlying these changes in choline transport have not been determined. However, possible mechanisms include changes in the number of transport proteins or changes in the turnover rate of existing transporters.

Taken together, these data imply HACT as a rate limiting and regulatory step in the synthesis of Ach. Further characterization and elucidation of the mechanisms by which HACT itself is regulated will greatly be accelerated by the purification of this protein and cloning of the gene responsible for its synthesis.

Choline acetyltransferase is the enzyme that catalyzes the synthesis of Ach from choline and acetyl-CoA (Fig. 1.2 and 1.3). ChAT was first purified from *Drosophila* (102). Purification of mammalian ChAT (103) proved more difficult due to its intrinsic instability and extremely low abundance in mammalian brain, estimated at 0.0001% of total brain protein. cDNAs have been cloned from *Drosophila* (104), pig (105), rat (106) and human (107, 108) coding for proteins of approximate molecular mass of 70 kDa. ChAT is generally thought of as a neuron-specific enzyme (13, 109, 110), although ChAT activity has been observed in non-neuronal tissue (111). A cell-specific silencer-element has been localized to a region distal to the ChAT promoter which restricts expression of the enzyme to cholinergic cells (112). Histochemical and immunocytochemical studies have shown that ChAT is distributed throughout all parts (cell body, dendrites, axon and axon terminals) of cholinergic neurons (113, 114). Subcellular fractionation studies of brain homogenates, however, suggest that the enzyme is enriched in nerve terminals (115, 116). ChAT is predominantly a cytosolic protein (117-119), although the presence of membrane-bound ChAT (10-20% of the enzyme) has been observed and been the subject of considerable

investigation (13, 110, 120). This membrane-associated ChAT is suggested to be a form of the enzyme coupled either to HACT (22) or to the biosynthesis, packaging and storage of Ach into synaptic vesicles (121). The significance of membrane-bound ChAT remains unclear but recent results indicate that the membrane form of ChAT is not necessary for the basal synthesis of Ach since depletion of greater than 90% of the membrane activity did not alter Ach synthesis (122).

It is generally believed that ChAT is not a rate-limiting factor in the synthesis of Ach. As mentioned previously, the stores of Ach in the terminals of cholinergic neurons are maintained at a near constant level under most physiological conditions. In agreement with this observation, the rate of synthesis of Ach has been found to adapt to its rate of release (13, 93). These findings suggest that the amount of ChAT in nerve terminals is sufficient to prevent a depletion of Ach levels during nerve stimulation (13, 120). The activity of ChAT assayed *in vitro* in the presence of saturating substrate concentrations greatly exceeds determinations of the rate of release of Ach *in vivo* (13). These findings should be interpreted with caution, however. The K_m values of ChAT for choline and acetyl-CoA are approximately 400 μM and 10 μM, respectively (13, 123). The enzyme, therefore, is unlikely to be operating at its maximal rate *in vivo* where the cytoplasmic concentrations of choline and acetyl-CoA have been estimated to be 50 and 5 μM, respectively (13). Thus, the *in vivo* rate of Ach release may be close to the activity of ChAT in the presence of physiological concentrations of substrate (13).

Modulation of ChAT specific activity has been demonstrated by treatment with a number of hormones and growth factors including nerve growth factor (124, 125), basic fibroblast growth factor (126, 127), ciliary neurotrophic factor (128), leukemia inhibitory factor (129) and retinoic acid (130, 131). The effect of nerve growth factor (132, 133), ciliary neurotrophic factor (134), leukemia inhibitory factor (133, 135) and retinoic acid (130) on ChAT activity has been shown to occur via increased expression of ChAT mRNA. In the case of nerve growth factor, elements within the ChAT gene have been identified which are required for NGF inducibility (112, 136). During development, environmental signals play an important role in the determination of the neurotransmitter phenotype of a neuron (137-139). For example, the sympathetic neurons innervating the sweat glands of rats undergo a switch in transmitter phenotype during postnatal development (reviewed in 137, 139). At birth, these neurons are adrenergic and, therefore, synthesize, store and release the neurotransmitter norepinephrine. A progressive decrease in the adrenergic properties accompanied by a progressive increase in cholinergic properties is observed in these neurons during postnatal development. In the adult, this population of sympathetic neurons is cholinergic and synthesizes and stores the neurotransmitter Ach. This change from an adrenergic to a cholinergic transmitter phenotype is called cholinergic switch or cholinergic differentiation and has been demonstrated to be induced by factors released by the innervated target tissue. The factors listed above, nerve growth factor, basic fibroblast growth factor, ciliary neurotrophic factor, leukemia inhibitory

factor and retinoic acid, can induce cholinergic differentiation of cultured sympathetic neurons (124-135) and, therefore, might be involved in target regulation of neurotransmitter phenotype during development *in vivo*.

1.2.4 Alternate Sources of Choline

Although extracellular choline is the primary source of choline for Ach synthesis, alternate sources of choline have been proposed. Choline mobilized by the action of phospholipases on membrane PC has been postulated as a potential source of choline for Ach synthesis under conditions where extracellular choline might be limiting, such as periods of intense synaptic activity (7). Consistent with this hypothesis is the finding that repeated depolarization of striatal slices (7, 8) causes a decrease in membrane PC which is rescued by choline administration (8). Evidence for Ach synthesis from choline derived from PC in neuronal cell lines has also been provided (9, 10). These findings have led to the suggestion that the selective vulnerability of cholinergic neurons in Alzheimer's disease and other cholinergic neurodegenerative disorders, might be related to the over-utilization of membrane PC as a source of choline for Ach synthesis (6). *De novo* synthesis of choline via the sequential methylation of phosphoethanolamine to phosphocholine (140) or phosphatidylethanolamine to PC (141) has been reported to occur in brain and, therefore, these compounds may also serve as sources of choline for Ach synthesis. The quantitative significance of these two pathways in cholinergic neurons, however, is questionable.

1.3 THESIS OBJECTIVE: PART I

In cholinergic neurons, choline is utilized not only for the synthesis of membrane PC, but also for the synthesis of the neurotransmitter Ach. The mechanisms that regulate the allocation of choline between these two end products have not been completely elucidated. Most of our knowledge on the regulation of Ach and PC synthesis in cholinergic neurons has been provided by studies examining choline metabolism either in synaptosomal fractions, isolated by subcellular fractionation of cholinergic tissue, or in neuroblastoma cell lines possessing cholinergic properties. These investigations have generally focused on determining the short-term mechanisms regulating Ach synthesis and the partitioning of choline between Ach and PC synthesis. In Chapter 2, choline metabolism in pure primary cultures of rat sympathetic neurons was examined in the context of cholinergic differentiation in order to determine the short-term and long-term mechanisms regulating the distribution of choline between Ach and PC synthesis.

In Chapter 3, the source of choline for Ach synthesis and the location of Ach synthesis were examined in isolated cell bodies and distal axons of cultured rat sympathetic neurons. Synthesis of Ach is believed to occur primarily in the axon terminals of cholinergic neurons from extracellular choline. Both ChAT and HACT have been found to be enriched in synaptosomal fractions isolated by subcellular fractionation and loss of ChAT and HACT has been observed in denervation studies

(see Section 1.2.3 above). Synthesis of Ach, however, has not been examined within isolated cell bodies or distal axons of cholinergic neurons in culture.

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Fig. 1.1 **Structure of a typical neuron**

A neuron is composed of 4 distinct regions: the cell body, dendrites, the axon and axon terminals. Signals are typically received on dendrites and on the cell body and travel down the axon to the axon terminals. A synapse is formed between axon terminals and effector cells, such as other neurons or muscle cells. A single axon can synapse to many effector cells. A neuron typically transmits its signal to an effector cell by releasing a neurotransmitter into the synapse which traverses the synapse and binds to receptors on the post synaptic membrane of the effector cell. Adapted from (139)

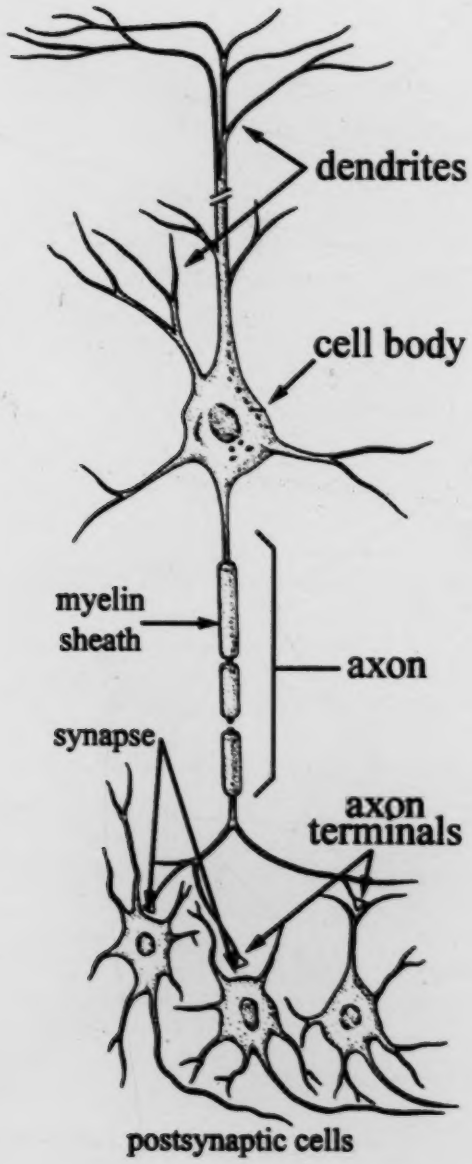

dendrites
cell body
myelin sheath
axon
synapse
axon terminals
postsynaptic cells

Fig. 1.2 **Structure of PC and Ach and their biosynthesis**

The synthesis of PC begins with the phosphorylation of choline to phosphocholine catalyzed by the enzyme choline kinase (CK). Subsequently, phosphocholine is activated by its conversion to cytidine diphosphocholine (CDP-choline), a reaction catalyzed by CTP:phosphocholine cytidylyltransferase (CT). The phosphocholine moiety of CDP-choline is then transferred to diacylglycerol leading to the formation of PC. This final step is catalyzed by cholinephosphotransferase (CPT). Ach synthesis is a one step reaction catalyzed by the enzyme choline acetyltransferase (ChAT).

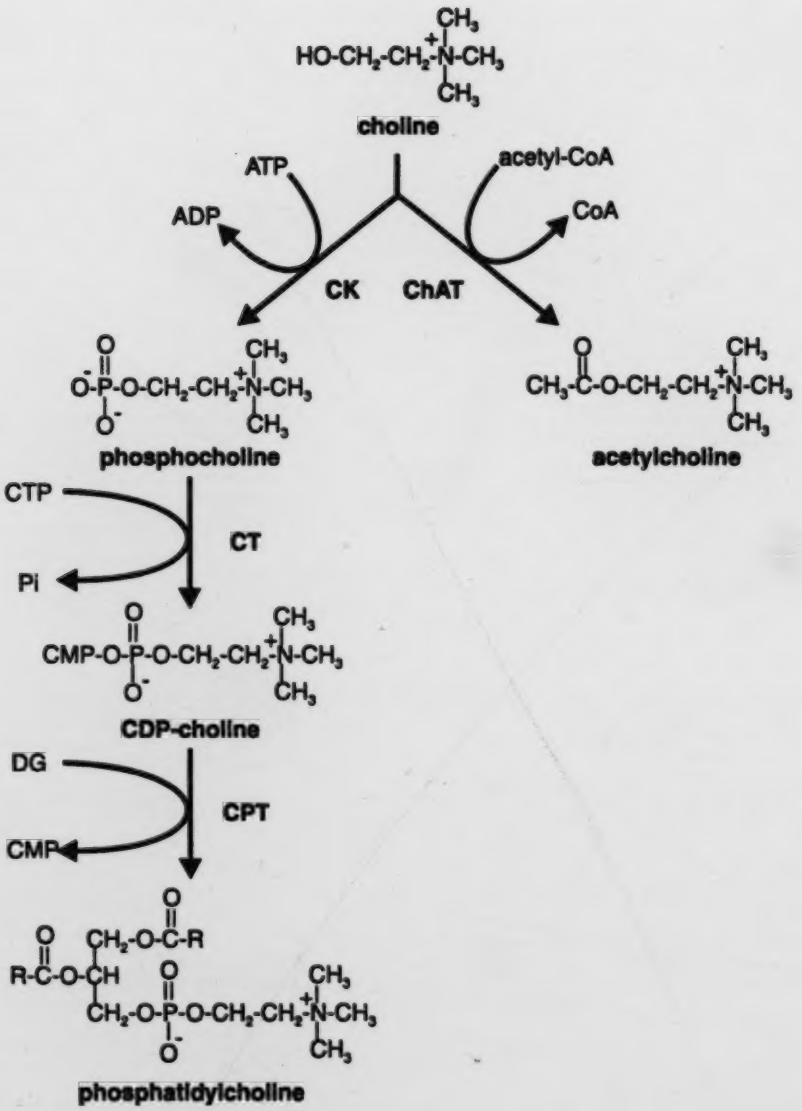
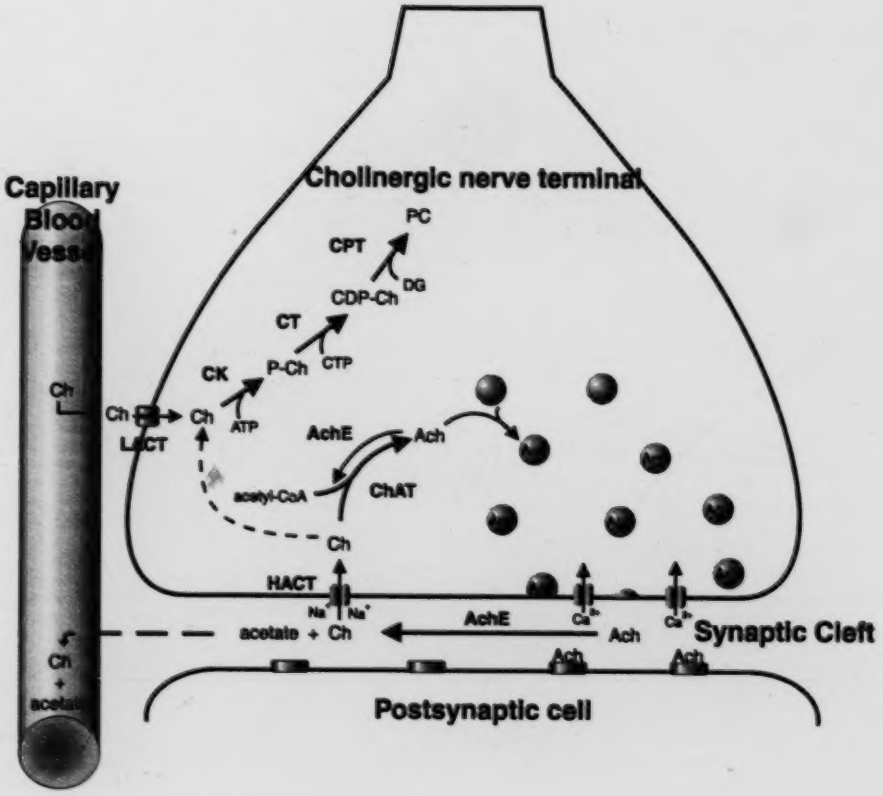

choline
ATP
ADP
CK
acetyl-CoA
CoA
ChAT
phosphocholine
acetylcholine
CTP
Pi
CT
CDP-choline
DG
CMP
CPT
phosphatidylcholine

Fig. 1.3 **Choline metabolism in a cholinergic nerve terminal**

Choline (Ch) from the blood mixes with the pool of choline in the extracellular fluid and enters neurons via low (LACT) and high (HACT) affinity choline transporters. Choline entering a neuron via LACT is used for membrane phosphatidylcholine (PC) synthesis. Choline taken up via HACT can be used for Ach or PC synthesis. Ach is stored in synaptic vesicles via the vesicular Ach transporter. Excess Ach is hydrolyzed by acetylcholinesterase (AchE) back to choline and acetate. Upon the arrival of an action potential to the axon terminal, voltage-gated Ca^{2+} channels open, Ca^{2+} enters the terminal and synaptic vesicles migrate and fuse to the synaptic membrane. Ach is released into the synaptic cleft, diffuses across the cleft and binds to receptors on the postsynaptic cell membrane. The signal is attenuated by the action of AchE, which hydrolyzes Ach to choline and acetate. 50-60% of the choline generated by AchE hydrolysis of released Ach is recycled for new Ach synthesis via HACT. CK, choline kinase; CT, CTP:phosphocholine cytidylyltransferase; CPT, cholinephosphotransferase; P-Ch, phosphocholine; CDP-Ch, cytidine diphosphocholine; ATP, adenosine triphosphate; CTP, cytidine triphosphate; DG, diacylglycerol; acetyl-CoA, acetyl-coenzyme A.


Capillary
Blood
Vessel
Cholinergic nerve terminal
PC
CPT
DG
CDP-Ch
CT
CTP
P-Ch
CK
ATP
Ch
Ch
Ch
HACT
AchE
Ach
acetyl-CoA
ChAT
Ch
HACT
Na+
Na+
AchE
acetate + Ch
Ca2+
Ca2+
Ach
Synaptic Cleft
Ach
Ach
Ch
+
Postsynaptic cell

CHAPTER 2

RECIPROCAL REGULATION OF CHOLINE ACETYLTRANSFERASE AND CHOLINE KINASE IN SYMPATHETIC NEURONS DURING CHOLINERGIC DIFFERENTIATION

The majority of this work was published in
Biochim. Biophys. Acta (1995) **1259**, 148-154

2.1 INTRODUCTION

Choline is the essential precursor in nucleated mammalian cells for the biosynthesis of PC, a major constituent of biological membranes. In cholinergic neurons, choline is also utilized for the biosynthesis of the neurotransmitter Ach. The following study was undertaken in order to obtain a better understanding of mechanisms which govern the distribution of choline between Ach and PC synthesis in cholinergic neurons.

Two choline transport systems have been identified in cholinergic neurons: low and high affinity (HACT) systems (1, 2). Evidence suggests that choline arising from the HACT is predominantly acetylated by the enzyme ChAT to generate Ach (3-6). Choline taken up via the low affinity system is not acetylated to Ach but is thought to be phosphorylated by the enzyme CK and used for the synthesis of PC (3, 4, 7). These observations imply that two pools of choline may exist in cholinergic neurons which are not equally available for acetylation by ChAT or phosphorylation by CK. Thus, one of the mechanisms regulating the distribution of choline between Ach and PC synthesis may be the supply of choline.

As described in Chapter 1 (Section 1.2.3), certain populations of neurons commonly undergo a change in neurotransmitter phenotype during development from adrenergic to cholinergic, called cholinergic differentiation. Adrenergic neurons secrete the neurotransmitter norepinephrine and cholinergic neurons secrete Ach. The sympathetic neurons innervating the sweat glands in the footpads of rats, for example,

undergo cholinergic differentiation upon innervation of their target (8). Other than an increase in ChAT activity, and thus of Ach synthesis, little is known about the changes in choline metabolism occurring in these neurons during the switch to a cholinergic phenotype. Widespread metabolic repercussions are likely since the cholinergic neuron must now divert choline, which solely supplies PC synthesis in the adrenergic state, into Ach as well as PC synthesis.

In order to examine the short-term and long-term mechanisms which regulate the distribution of choline between Ach and PC synthesis in cholinergic neurons, primary sympathetic neurons were utilized because of their ability to change transmitter phenotype in culture in the presence of a differentiation factor. The results using this model system confirm that choline, arising from HACT, may be utilized as a substrate for the synthesis of PC as well as Ach in cholinergic rat sympathetic neurons. In addition, the results provide evidence that Ach and PC biosynthesis are reciprocally regulated during cholinergic differentiation.

2,2 Materials and Methods

[*methyl*-^{3}H]Choline chloride (15 Ci/mmol) and [^{3}H]acetyl-CoA (3.4 Ci/mmol) were purchased from Amersham Canada, Oakville, ON. Thin-layer chromatography plates (glass silica gel G plates, 0.25 mm thickness, and plastic cellulose plates, 0.1 mm thickness) were obtained from BDH, Edmonton, Canada. Eserine sulfate, HC-3, all-*trans* RA, acetyl-CoA, Ach, choline, phosphocholine, cytidine 5'-diphosphocholine,

PC were purchased from Sigma (St. Louis, MO.). All other reagents and chemicals were from Sigma or Fisher Scientific.

Preparation of neuronal cultures

General procedures for culture of rat sympathetic neurons have been previously described (9). Briefly, superior cervical ganglia were dissected from newborn Sprague-Dawley rat pups (supplied by the University of Alberta farm). The ganglia were chemically dissociated by separate incubations in 0.1% trypsin and 10 μg/ml DNAse followed by mechanical dissociation (9). Cells were plated on rat tail collagen-coated 24-well plates at a concentration of 2 ganglia/well. General culture procedures essentially followed the methods of Hawrot and Patterson (10). The standard culture medium was L15 medium (GIBCO, Grand Island, NY) supplemented with the prescribed additives including bicarbonate and 6% methylcellulose. The plating medium consisted of standard L15CO_2 supplemented with 100 ng/ml 2.5S nerve growth factor (NGF) (Cedarlane Laboratories, Hornby, ON, Canada), 2.5% rat serum (provided by University of Alberta Lab Animal Services), 1 mg/ml ascorbic acid and 10 μM cytosine arabinoside to eliminate non-neuronal cells. After 7 days fresh medium containing NGF, rat serum and ascorbic acid was added to the cultures. All cultures were incubated in a 5% CO_2 atmosphere at 37°C and were fed as required every 4-6 d. 10 d old cultures were used as the starting point for further treatments. Choline-free medium consisted of L15 medium containing all the ingredients listed by GIBCO, but with choline omitted. 50 mM K^+-medium consisted of L15 medium

containing all the ingredients listed by GIBCO, but with KCl elevated to 50 mM and the NaCl concentration adjusted to maintain osmolarity.

Enzyme Assays

Sympathetic neuron cultures were washed 3 times with ice-cold phosphate-buffered saline, harvested in ice-cold homogenization buffer (0.5% Triton X-100 in sodium phosphate buffer, pH 6.8) and sonicated. Aliquots of the sonicate were used for protein quantification and measurement of ChAT and CK activities.

ChAT activity was determined according to Fonnum (11). The acetyl-CoA concentration was 135 μM and incubations were performed for 30 min. ChAT activity was expressed as nmol/min per mg of protein.

CK activity was determined by the method of Weinhold and Rethy (12) and was expressed as nmol/min per mg protein.

Incorporation of radiolabeled precursor into Ach, PC and intermediates of PC biosynthesis

Cells were incubated for 2.5 h at 37°C in a 5% CO_2 incubator in choline-free, methylcellulose-free L15CO_2 medium supplemented with NGF, rat serum, ascorbic acid, 10 μCi/well [*methyl*-^{3}H]choline (15 Ci/mmol) and other supplements as indicated. The choline concentration in the medium was approximately 3.5 μM. Cells

were washed 3 times with ice-cold phosphate-buffered saline, harvested in ice-cold water and sonicated. Both the washing solution and the water used for harvesting contained 15 µg/ml eserine, an inhibitor of the enzyme acetylcholinesterase which hydrolyzes Ach to acetate and choline. An aliquot of the sonicate was taken for protein determination. To the remainder of the sample, chloroform, methanol and water were added to give a final ratio of chloroform/methanol/water of 2:1:1 (v/v). The samples were extracted according to the procedure of Folch *et al.* (13).

For separation of the water-soluble metabolites, the aqueous extracts were evaporated to dryness and dissolved in an aqueous solution containing 1 mg/ml choline and 1 mg/ml Ach. The samples were applied to cellulose thin layer chromatography plates which had been pre-spotted with cytidine 5'-diphosphocholine (25 mg/ml) and phosphocholine (50 mg/ml) carrier. The aqueous components were separated in the solvent system *n*-butanol/95% ethanol/acetic acid/water (70:21:10:29, v/v) essentially according to Marchbanks and Israel (14). Bands corresponding to authentic standards of Ach, choline, phosphocholine and CDP-choline were scraped from the plates and the radioactivity incorporated was measured by liquid scintillation counting.

PC was isolated by applying the chloroform phase to thin-layer chromatography (silica plates) and the plates developed in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, v/v). The band

corresponding to authentic PC was scraped from the plates and radioactivity was measured by liquid scintillation counting.

Choline Uptake Assay

Choline uptake assays were performed essentially as described by Rylett *et al.* (15). Cultures were washed 3 times with 37°C Krebs-Ringer buffer (124 mM NaCl, 5.0 mM KCl, 1.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 20 mM HEPES-NaOH (pH 7.4) and 10 mM glucose). Choline uptake was initiated by addition of Krebs-Ringer buffer containing 2 μCi [*methyl*-^{3}H]choline (1 μM; 15 Ci/mmol), and incubation was continued at 37°C for 5 min. Uptake was terminated by aspiration of the incubation solution, rapid washing with 1 ml of ice-cold Krebs-Ringer buffer 3 times (completion of all 3 washes required less than 20 s) and the cells were resuspended in 0.1 M NaOH. Aliquots were then taken for determination of the amount of radioactivity and protein. Parallel cultures were incubated with [*methyl*-^{3}H]choline in the presence of 10 μM HC-3 or in sodium-free Krebs-Ringer buffer in which NaCl was replaced iso-osmotically with LiCl and HEPES-NaOH was replaced with HEPES-Tris. Choline uptake was expressed as picomoles of choline incorporated per mg of protein per 5 min.

Protein Determination

Protein amount was determined using the micro version of the bicinchoninic acid procedure (Pierce kit), using bovine serum albumin as the standard.

2.3 Results

2.3.1 Effect of retinoic acid treatment on Ach and PC biosynthesis

In order to study the metabolic changes that occur during cholinergic differentiation, sympathetic neurons were cultured in the presence of 5 μM RA for up to 12 d. These conditions have previously been shown to induce ChAT activity in sympathetic neurons (16, 17). To test the hypothesis that the activities of ChAT and CK are inversely regulated during cholinergic differentiation, the specific activities of ChAT and CK were examined in sympathetic neuron cultures which had been treated for different lengths of time with 5 μM RA. ChAT specific activity increased as the time of treatment with RA was extended (Fig. 2.1). In the representative experiment depicted in Fig. 2.1, the specific activity of ChAT increased from 1.4 nmol/min/mg protein to 2.6 nmol/min/mg protein after 12 days of treatment. The basal ChAT activity of untreated sympathetic neurons varied from preparation to preparation. Although the absolute changes in specific activity upon cholinergic differentiation were similar, the fold increase in ChAT specific activity varied from 2- to 4-fold over the treatment period depending on the basal ChAT activity. Consistent with our hypothesis, a decrease in the specific activity of CK was observed from 2.0 nmol/min/mg protein to 1.3 nmol/min/mg protein (Fig. 2.1). Similar results were

obtained when leukemia inhibitory factor was used as the cholinergic differentiation factor instead of RA (Fig. 2.2).

Next, it was determined if the changes in the specific activities of ChAT and CK were accompanied by changes in the radioactive labeling of metabolites of these two biosynthetic pathways. Cultures which had been treated for various times with RA were pulsed for 2.5 h with [^{3}H]choline. Neurons were harvested and the radioactivity incorporated into the aqueous and organic metabolites was measured. Incorporation of [^{3}H]choline into Ach increased 2-fold after 12 d of RA treatment (Fig. 2.3A), consistent with the changes in the specific activity of ChAT previously observed (Fig. 2.1). The amount of label incorporated into Ach is the net result of synthesis and degradation. In a separate experiment 50 μM eserine, an inhibitor of acetylcholinesterase which catalyzes the degradation of Ach, was added during the pulse with [^{3}H]choline. Approximately 2.5-fold more radioactivity was recovered in Ach (Table 2.1), indicating that active degradation of Ach occurred in these neurons.

No significant change in the incorporation of [^{3}H]choline into phosphocholine was observed (Fig. 2.3B). It is likely that any slight changes occurring in the size of the phosphocholine pool might be masked due to the large size of the phosphocholine pool. Furthermore, no significant changes were observed in the incorporation of [^{3}H]choline into either CDP-choline (Fig. 2.3B) or PC (Fig. 2.3C) or in the total

amount of radioactivity taken up by RA-treated neurons versus control neurons (Fig. 2.3D).

2.3.2 RA treatment does not affect HACT

Two hallmarks of cholinergicity are: the presence of ChAT enzyme and sodium-dependent, HC-3-sensitive HACT (3-6, 18). It is generally believed that the rate of Ach synthesis is governed by the supply of choline and, thus, by HACT, which is the primary source of choline for Ach synthesis (3-6, 18). Cultured rat sympathetic neurons exhibit both high and low affinity choline transport (19). The effect of RA on HACT was therefore determined. The sensitivity of choline uptake and incorporation by cultures of rat sympathetic neurons to inhibition by HC-3 was first determined. Cultures were labeled for 2.5 h with [^{3}H]choline in the presence of various concentrations of HC-3 and the radioactivity incorporated into the various metabolites was measured. 10 μM and 50 μM HC-3 reduced the incorporation of radiolabel into Ach (Fig. 2.4A) by approximately 30% and 45%, respectively. Similar decreases in radiolabel incorporated into the various metabolites of the PC biosynthetic pathway (Fig. 2.4B and 2.4C) were observed.

To determine if RA modulates HACT, RA-treated cultures and untreated controls were labeled for 5 min with [^{3}H]choline either in the presence or absence of 10 μM HC-3 or in Na^+-free buffer. Choline uptake of both untreated and RA-treated neuron cultures was dramatically reduced in the absence of Na^+ or in the presence of 10 μM

HC-3 (Fig. 2.5). No significant differences in choline uptake were observed, however, between untreated and RA-treated cultures. These results suggest that RA does not affect HACT.

2.3.3 The HACT system supplies choline for Ach and PC synthesis in sympathetic neurons

The HACT system is believed to be linked to Ach synthesis in cholinergic neurons (3-5). In synaptosomal preparations, inhibition of HACT using HC-3 or Na^+-free conditions dramatically affected Ach synthesis but only marginally affected phosphocholine and PC synthesis (1, 2, 20, 21). These results provide evidence for a link between HACT and Ach synthesis. A low affinity, Na^+-independent, HC-3-insensitive choline uptake system is thought to deliver choline for membrane PC synthesis in cholinergic neurons (3, 4, 7). When the incorporation of [^{3}H]choline into Ach in RA-treated sympathetic neurons was determined under normal and Na^+-free conditions, a 91% decrease in the amount of radiolabel incorporated into Ach (3.5 μM external choline) was observed by removal of Na^+ from the incubation medium (Fig. 2.6A). A greatly decreased incorporation of [^{3}H]choline into Ach was observed even when the extracellular choline concentration was raised to 100 μM. These results support the hypothesis that a high affinity, Na^+-dependent transporter supplies choline for Ach synthesis since previous studies have demonstrated that ChAT activity is unaffected by removal of Na^+ (1, 2, 21). These results do not rule out the possibility that at elevated external choline concentrations (>20 μM), a small amount of choline

delivered via a low affinity system might also be utilized for Ach synthesis. The absence of sodium in the incubation medium significantly reduced the incorporation of radiolabel into phosphocholine. Incorporation of [^{3}H]choline into phosphocholine was decreased by 52% at 3.5 μM choline and 41% at 20 and 100 μM choline (Fig. 2.6B). These results suggest that choline utilized for phosphocholine synthesis, and consequently for PC synthesis, arises from both low and high affinity choline transport. In parallel experiments, the effect of 10 μM HC-3 on the incorporation of [^{3}H]choline into RA-treated sympathetic neurons was determined (Table 2.2). HC-3 inhibited the incorporation of radiolabel into both Ach and phosphocholine but the extent of inhibition was dependent on the extracellular concentration of choline. HC-3 had little effect on [^{3}H]choline incorporation into Ach and phosphocholine at elevated concentrations of extracellullar choline.

The effect of HC-3 treatment or removal of Na^+ from the incubation medium on CK enzyme activity was also determined. Cultures of sympathetic neurons were washed and incubated for 2.5 h in the presence of either 10 μM or 50 μM HC-3 or in the absence of Na^+. The extracellular choline concentration was 3.5 μM. The specific activity of CK in untreated cultures of sympathetic neurons was 3.15 ± 0.46 nmol/min/mg. No significant effect on CK activity was observed by treatment of neurons with 10 μM HC-3 (3.13 ± 0.96 nmol/min/mg) or in the absence of Na^+ (2.82 ± 0.50 nmol/min/mg). However, a 33% inhibition of CK activity was observed in the presence of 50 μM HC-3 (2.11 ± 0.46 nmol/min/mg). Thus, the reduced incorporation

of [^{3}H]choline into phosphocholine in the presence of high concentrations of HC-3 (Fig. 2.4) is due not only to inhibition of choline transport but also to inhibition of CK.

2.3.4 Long-term treatment with 50 mM K^+ inhibits ChAT specific activity and increases CK specific activity

Depolarization is thought to be one of the factors which affects the cholinergic/adrenergic status of sympathetic neurons (22, 23). Long-term treatment of sympathetic neurons with elevated K^+ concentrations has been shown to suppress the increase in ChAT specific activity induced by cholinergic differentiation factors (22, 23). The effect of long-term treatment of sympathetic neurons with elevated K^+ on CK activity was, therefore, determined. Fig. 2.7 shows that, while ChAT activity was suppressed by up to 90% upon incubation with 50 mM K^+, CK activity was stimulated by approximately 1.3-fold.

2.4 Discussion

This study examined the regulation of the distribution of choline between two key biosynthetic pathways of cholinergic neurons, Ach and PC synthesis. Evidence was provided that: (a) During the process of cholinergic differentiation induced by RA, ChAT enzyme activity is enhanced leading to increased synthesis of Ach, whereas CK activity is decreased without affecting overall PC synthesis. (b) Both ChAT and CK utilize choline transported by HACT. Choline taken up by the low affinity transporter

appears to be used exclusively for PC synthesis. (c) Long-term treatment of sympathetic neurons with 50 mM K^+ has the opposite effect on the activities of these two enzymes compared to RA treatment; CK activity is increased and ChAT activity is decreased.

As described in Chapter 1 and the introduction to this Chapter, many investigations have demonstrated the existence of two choline transporters in cholinergic neurons with high and low affinity for choline (3-5). Choline taken up via the high affinity transporter is thought to be the predominant source of choline for Ach synthesis (3-5) and is characterized by its Na^+-dependency and sensitivity to inhibition by HC-3. Low affinity choline transport is not dependent on Na^+, is less sensitive to inhibition by HC-3 and supplies choline for synthesis of membrane PC (3, 4, 7). The data presented in this Chapter are consistent with these studies. Incorporation of [^{3}H]choline into Ach was inhibited 90% by removal of Na^+ from the incubation medium (Fig. 2.6A) and 47% by treatment with 10 μM HC-3 (Table 2.2) at an extracellular choline concentration of 3.5 μM. Raising the extracellular concentration of choline had little effect on the incorporation of radiolabel into Ach when Na^+ was absent from the medium (Fig. 2.6A). HC-3 is a reversible competitive inhibitor of choline uptake (4) and therefore raising the extracellular choline concentration abolished the inhibitory effect of HC-3 on Ach synthesis (Table 2.2). Considerable phosphocholine synthesis occurred in the absence of Na^+ (Fig. 2.6B) or presence of HC-3 (Table 2.2) at both low and high concentrations of extracellular choline. These results suggest that

choline used for phosphocholine synthesis, and consequently for PC synthesis, is taken up both by low and high affinity choline transport. Similar results have been obtained in synaptosomal preparations (1, 2, 20, 21) and in sympathetic neurons (19). Since several isoforms of CK have been identified (24, 25), it is possible that a specific CK isozyme synthesizes phosphocholine from choline taken up by HACT and this isozyme of CK is regulated by RA.

Two characteristics which have been used to define cholinergicity are high levels of ChAT enzyme activity and the presence of HACT. An increase in the activity of either of these systems causes an increase in Ach synthesis (6, 18 and see Chapter 1 Section 1.2.3). Although RA increased ChAT activity, HACT was not stimulated by RA-treatment in cultured sympathetic neurons (Fig. 2.5). HACT was observed in non-RA-treated sympathetic neurons, as demonstrated by a dramatic decrease in choline uptake by untreated sympathetic neurons in the presence of HC-3 or in the absence of Na^+ (Fig. 2.5). Since sympathetic neurons are dependent on the presence of NGF for survival and NGF has been demonstrated to increase the cholinergic properties of certain populations of neurons (26-28), it is possible that NGF in the culture medium induces HACT in these cultures. The cholinergic properties of sympathetic neurons also increase with increasing age in culture (19). Alternatively, the presence of HACT in sympathetic neurons may be due to the presence of serum factors which might induce some cholinergic differentiation (29).

The hypothesis in this study was that cholinergic differentiation impinges on the regulation of PC biosynthesis. In testing this hypothesis, it was demonstrated that cholinergic differentiation, induced by RA or leukemia inhibitory factor, decreased the specific activity of choline kinase (Fig. 2.1 and 2.2), the enzyme which utilizes choline as a substrate and catalyzes the first step in the *de novo* biosynthesis of PC. A reciprocal relationship between the activities of ChAT and CK during cholinergic differentiation was observed. Further support of this hypothesis is provided by the long-term treatment of sympathetic neurons with 50 mM K^+. This treatment decreases ChAT activity and increases CK activity (Fig. 2.7A and 2.7B), the reverse of the effect seen with RA. Both RA and leukemia inhibitory factor increase the amount of ChAT mRNA and thereby cause the cholinergic differentiation of sympathetic neurons (17, 30). Since RA treatment increases ChAT mRNA levels, it is reasonable to speculate that the effect of RA on CK may also occur at the mRNA level. More specifically, RA may modulate the mRNA levels of a specific isozyme of CK which is linked to the synthesis of phosphocholine via HACT, as suggested above. Unfortunately, the technical difficulties involved in reliably detecting modest changes in mRNA levels with small quantities of tissue, prevented us from testing this hypothesis.

Suidan and Tolkovsky (19) have suggested that cultured sympathetic neurons are poised to allocate choline symmetrically between Ach and PC synthesis at their growing neurite endings. Recent studies have demonstrated that significant quantities

of PC are synthesized locally in the axons of sympathetic neurons and that this axonal synthesis of PC is crucial for normal axonal growth (31-33). Since the HACT system is also believed to be localized to axon terminals (34, 35), our results are in agreement with the hypothesis put forth by Suidan and Tolkovsky (19); choline taken up via the Na^+-dependent, HC-3-sensitive HACT system in axon terminals can be acetylated or phosphorylated (Fig. 2.6 and Table 2.2).

A potential model of choline utilization in sympathetic neurons is illustrated in Fig. 2.8. In cholinergic neurons, choline is taken up via high and low affinity uptake mechanisms. PC is synthesized primarily via choline taken up by low affinity choline transport. Choline taken up via HACT is available to both ChAT and CK. Thus, this source of choline is utilized for the synthesis of Ach as well as PC. It is possible that different isozymes of CK may utilize choline taken up via the high or low affinity choline uptake systems. Alternatively, the situation depicted in Fig. 2.8 might be representative of choline metabolism in axon terminals, specifically. Thus, only choline kinase located within axon terminals competes for choline taken up via HACT.

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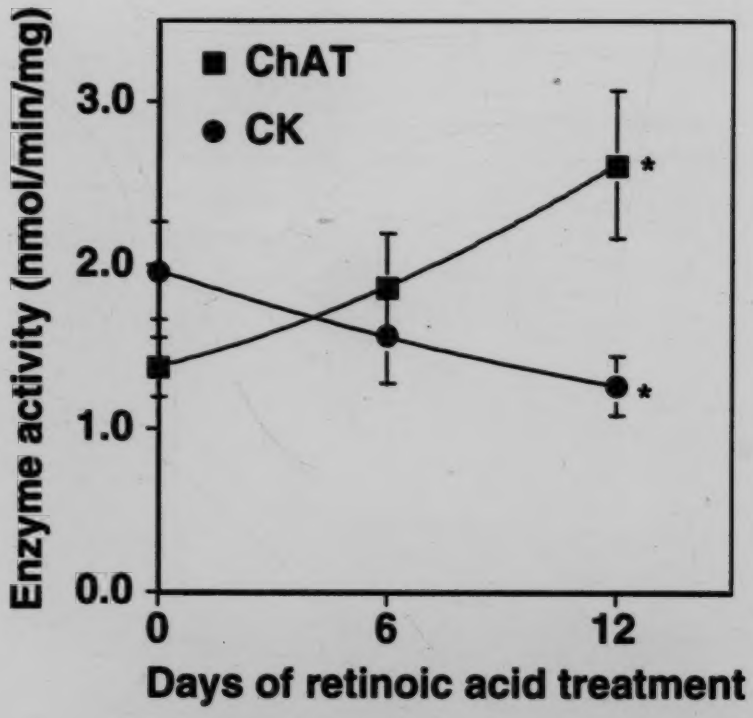


Fig. 2.1 **Retinoic acid treatment increases ChAT activity and decreases CK activity in sympathetic neurons.**

Sympathetic neurons were treated with 5 μM retinoic acid for 0, 6 or 12 days prior to the start of the experiment. Equal aliquots from the cultures were assayed for protein, ChAT activity (squares) and CK activity (circles). Each value represents the mean ± S.D. of 6 culture dishes. The experiment was repeated twice with similar results. Significantly different from control (no treatment), * $P<0.05$.

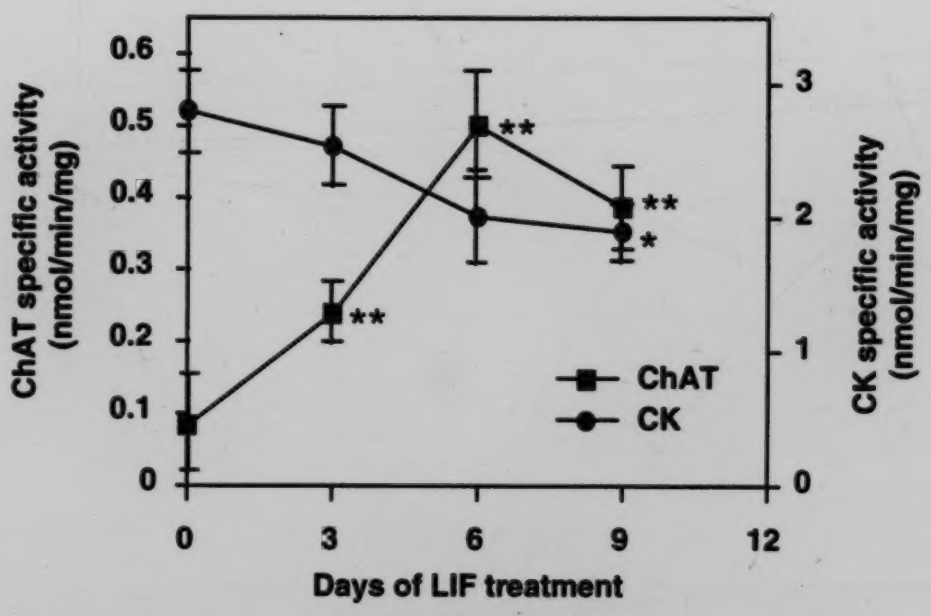


Fig. 2.2 **Leukemia inhibitory factor treatment increases ChAT activity and decreases CK activity in sympathetic neurons**

Sympathetic neurons were treated with 10 ng/ml leukemia inhibitory factor for 0, 6 or 9 days prior to the start of the experiment. Equal aliquots from the cultures were assayed for protein, ChAT activity (squares) and CK activity (circles). Each value represents the mean ± S.D. of 5 culture dishes. The experiment was repeated twice with similar results. Significantly different from control (no treatment), * $P<0.01$, **$P<0.001$.

Fig. 2.3 **The effect of retinoic acid on the incorporation of [^{3}H]choline into sympathetic neurons.**

Sympathetic neurons were treated with 5 μM retinoic acid for 0, 6 or 12 days prior to the start of the experiment. Cultures were incubated for 2.5 h in medium containing 10 μCi/ml [^{3}H]choline (3.5 μM). Incorporation into: panel A, Ach; panel B, choline, p-choline (phosphocholine), CDP-choline (cytidine diphosphocholine); panel C, PC; panel D, total incorporation into aqueous and organic compounds and total incorporation (sum of total incorporation into aqueous and organic compounds) was determined. Each value represents the mean ± S.D. of 6 culture dishes. The experiment was repeated twice with similar results.

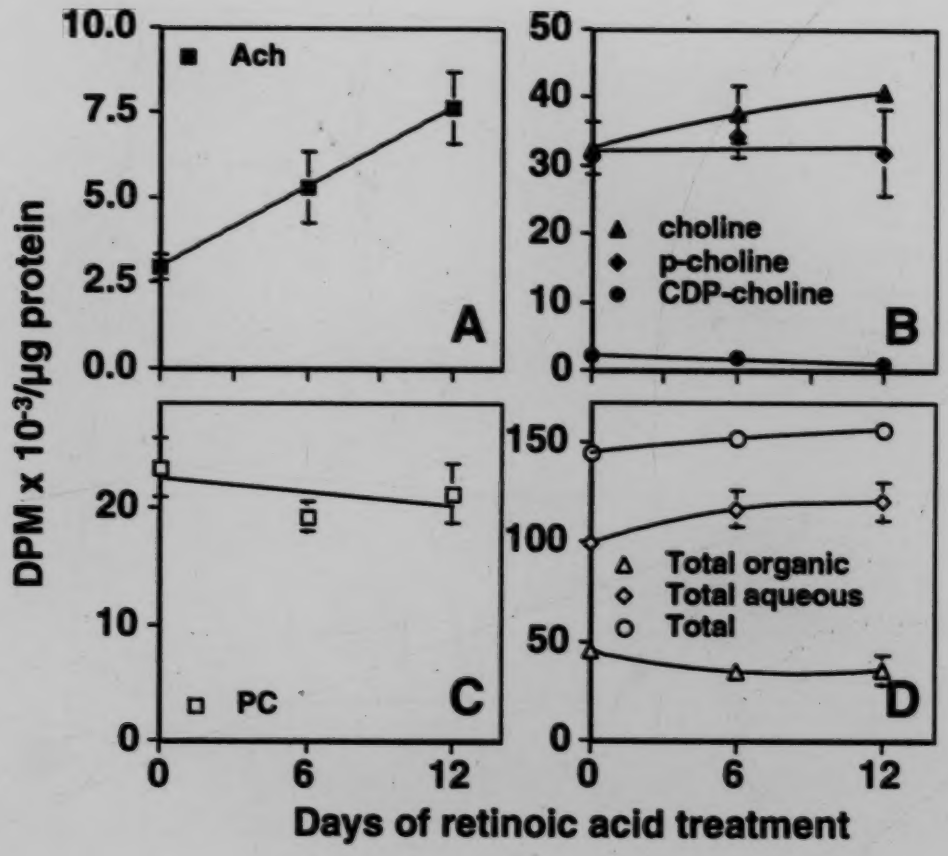

10.0
7.5
5.0
2.5
0.0
Ach
A
50
40
30
20
10
0
choline
p-choline
CDP-choline
B
20
10
0
PC
C
150
100
50
0
Total organic
Total aqueous
Total
D
0
6
12
0
6
12
DPM x 10-3/µg protein
Days of retinoic acid treatment

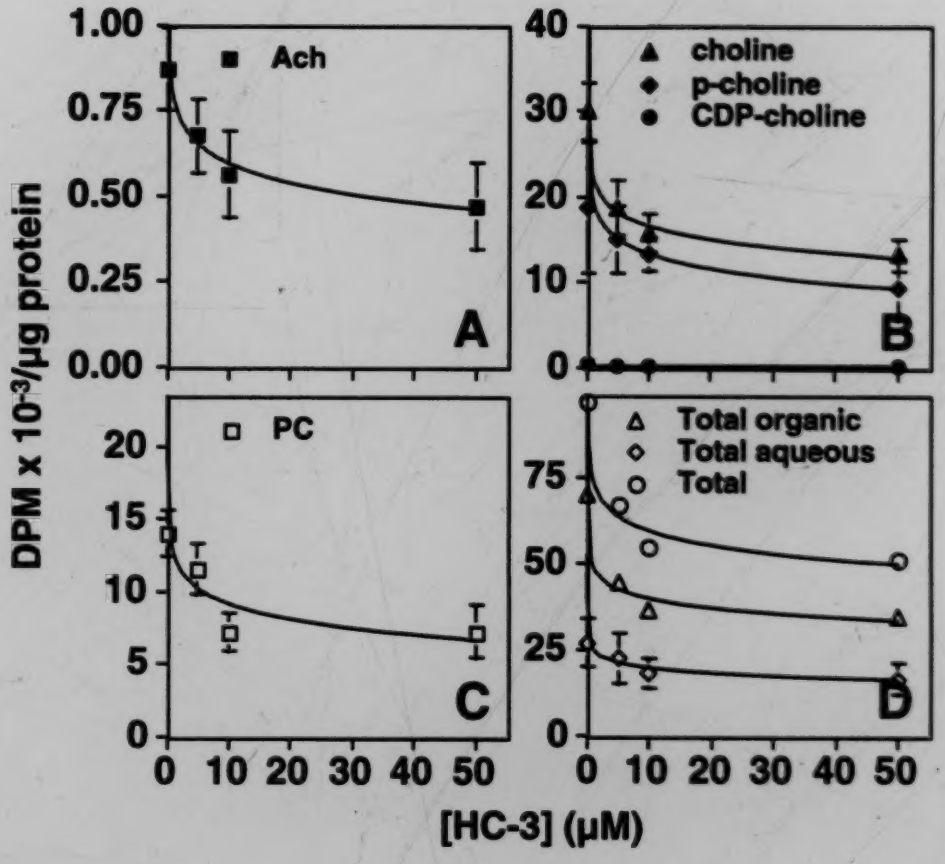
Table 2.1 **Eserine treatment increases [^{3}H]choline recovered in acetylcholine**

Sympathetic neurons were treated with 5 μM retinoic acid for 0, 6 or 12 days prior to the start of the experiment. Cultures were radiolabeled with 10 μCi/ml [^{3}H]choline for 2 h in the absence or presence of 50 μM eserine. The incorporation of [^{3}H]choline into Ach, choline, phosphocholine (p-choline), CDP-choline and PC (phosphatidylcholine) was determined. Data are dpm recovered per μg of total cell protein and are averages ± S.D. for four individual cultures. The experiment was repeated once with similar results. Significantly different from control (no treatment), * P<0.002, **P<0.0001.

Days of RA treatment	Ach	choline	p-choline	CDP-choline	PC
	(DPM x 10^{-3}/μg protein)				
- eserine					
0	1.4 ± 0.3	19.5 ± 4.9	15.2 ± 2.3	3.2 ± 1.0	8.8 ± 0.8
6	1.9 ± 0.6	16.3 ± 2.5	12.8 ± 1.5	1.9 ± 0.5	7.7 ± 0.8
12	3.9 ± 0.7	26.0 ± 0.6	13.6 ± 2.1	1.2 ± 0.1	7.5 ± 0.5
+ eserine					
0	*3.7 ± 0.6	24.5 ± 2.6	17.9 ± 1.9	3.1 ± 0.6	8.5 ± 0.6
6	*4.2 ± 0.3	28.0 ± 1.1	16.3 ± 0.7	1.9 ± 0.2	6.6 ± 0.5
12	**11.9 ± 1.2	33.2 ± 4.3	13.9 ± 1.8	1.3 ± 0.1	7.8 ± 0.9

Fig. 2.4 **Concentration curve of the effect of HC-3 on the incorporation of [^{3}H]choline into sympathetic neurons.**

Sympathetic neurons were treated with 5 μM retinoic acid for 12 days prior to the start of the experiment. The cultures were incubated for 2.5 h with 10 μCi [^{3}H]choline in the presence of HC-3 at the indicated concentrations. Incorporation into: panel A, Ach; panel B, choline, p-choline (phosphocholine), CDP-choline (cytidine diphosphocholine); panel C, PC; panel D, total incorporation into aqueous and organic compounds and total incorporation (sum of total incorporation into aqueous and organic compounds) was determined. Each value represents the mean ± S.D. of 4 culture dishes. The experiment was repeated twice with similar results.


DPM x 10-3/µg protein
1.00
0.75
0.50
0.25
0.00
Ach
A
40
30
20
10
0
choline
p-choline
CDP-choline
B
20
15
10
5
0
PC
C
75
50
25
0
Total organic
Total aqueous
Total
D
0 10 20 30 40 50
0 10 20 30 40 50
[HC-3] (µM)

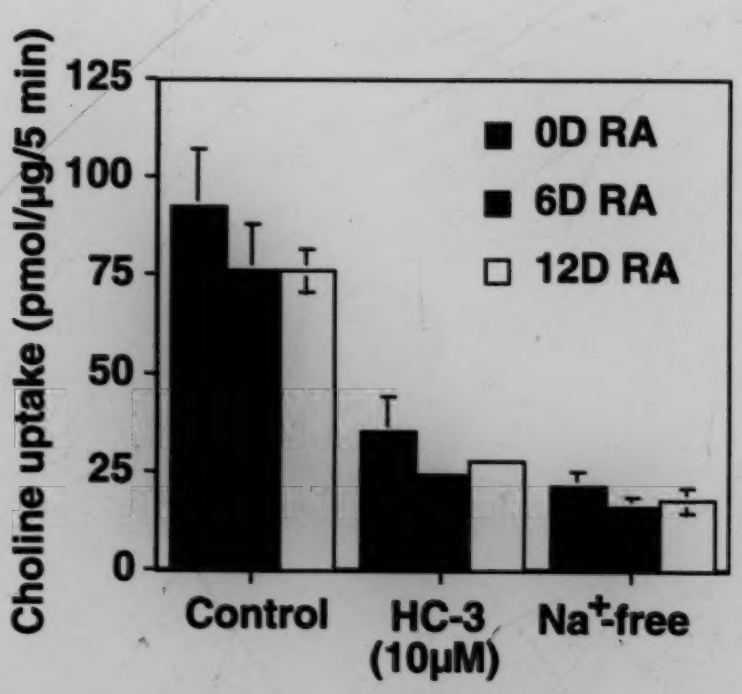


Fig. 2.5 **Retinoic acid treatment does not affect choline uptake.**

Sympathetic neurons were treated with 5 µM retinoic acid for 0 (solid bars), 6 (shaded bars) or 12 (open bars) days prior to the start of the experiment. Cultures were incubated for 5 min in Krebs-Ringer buffer containing 2 µCi/well [^{3}H]choline (1 µM). The total radioactivity incorporated into the cells was determined in control incubations (without HC-3 and in the presence of Na^+) and incubations in the presence of 10 µM HC-3 or in Na^+-free buffer. Each value represents the mean ± S.D. of 4 culture dishes. The experiment was repeated twice with similar results.

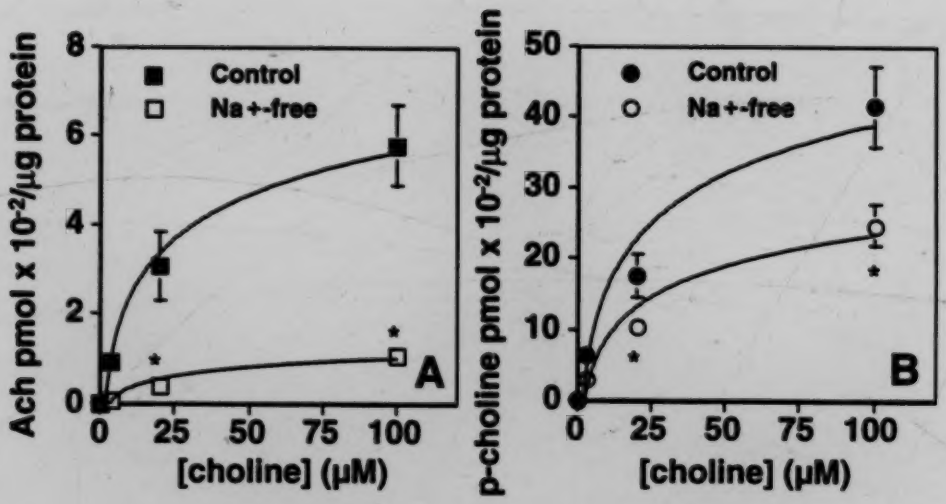


Fig. 2.6 **ChAT and CK have access to the same cellular pool of choline.**

Sympathetic neurons were treated with 5 µM retinoic acid for 12 days prior to the start of the experiment. Cultures were then washed and incubated for 2.5 h in Krebs-Ringer buffer (control, solid symbols) containing 10 µCi/ml [^{3}H]choline at the indicated concentrations of choline. Parallel incubations were performed in Na^+-free buffer (open symbols). Radioactivity incorporated into Ach (panel A) and phosphocholine (panel B; p-choline) was determined. Each value represents the mean ± S.D. of 4 culture dishes. The experiment was repeated twice with similar results. Significantly different from control (no treatment), * $P<0.05$.

Table 2.2 **HC-3 inhibits the incorporation of [^{3}H]choline into Ach and phosphocholine**

Sympathetic neurons were treated with 5 μM retinoic acid for 12 days prior to the start of the experiment. Cultures were then washed and incubated for 2.5 h in medium containing 10 μCi/ml [^{3}H]choline at the indicated concentrations of choline. Parallel incubations were performed in the presence of 10 μM HC-3. The radioactivity incorporated into Ach and p-choline (phosphocholine) was determined. Each value represents the mean ± S.D. of 4 culture dishes. The experiment was repeated once with similar results. Significantly different from control (no treatment), * $P<0.05$, ** $P<0.001$

[choline]	Ach	p-choline
(μM)	(pmol/μg protein)	
- HC-3		
3.5	61 ± 22	1497 ± 261
20	240 ± 83	5268 ± 1240
100	419 ± 47	11587 ± 793
+ HC-3		
3.5	*32 ± 11	**1075 ± 107
20	*163 ± 35	4307 ± 793
100	385 ± 103	9381 ± 1649

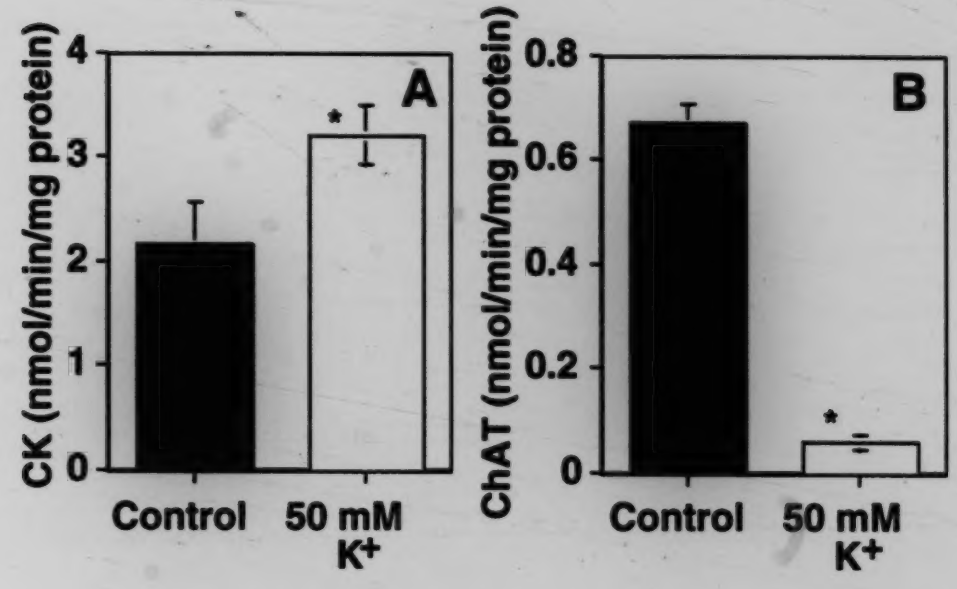


Fig. 2.7 **50 mM K^+ decreases ChAT activity and increases CK activity in sympathetic neurons.**

Sympathetic neurons were cultured for 12 days in $L15CO_2$ medium (solid bars) or in $L15CO_2$ medium in which the KCl concentration was elevated to 50 mM (open bars) (NaCl concentration adjusted to maintain osmolarity) prior to the start of the experiment. CK specific activity (panel A) and ChAT specific activity (panel B) were assayed in aliquots of cellular sonicates. Each value represents the mean ± S.D. of 6 culture dishes. The experiment was repeated twice with similar results. Significantly different from control, * $P<0.05$.

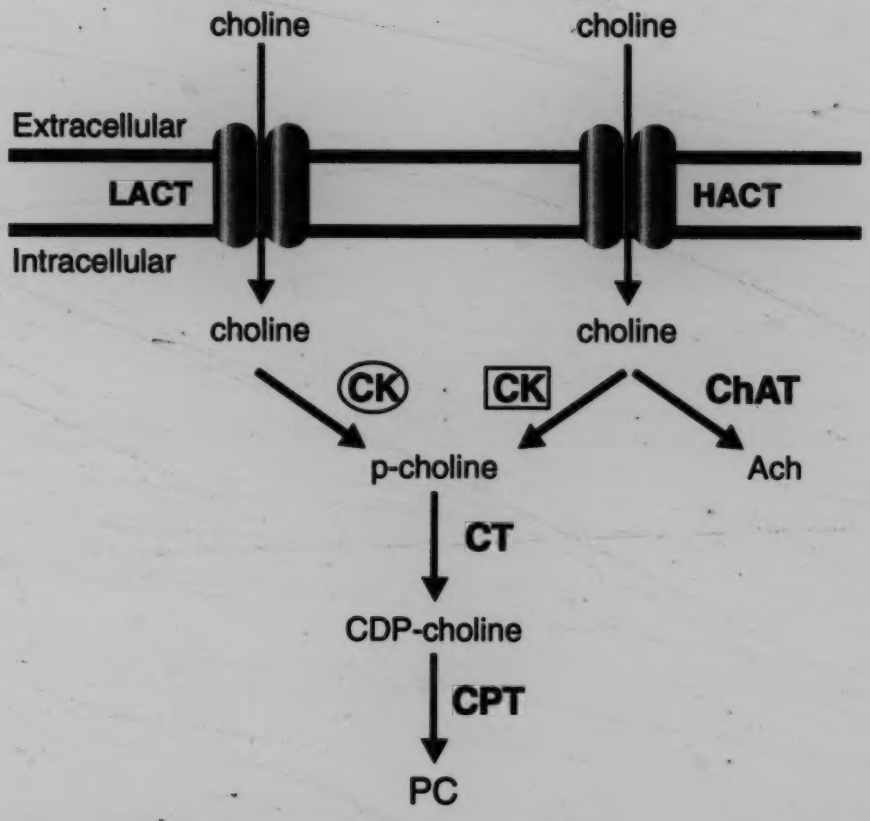


Fig. 2.8 **Model of choline utilization in sympathetic neurons expressing cholinergic properties.**

See text (Section 2.5) for description. LACT, low affinity choline transport; HACT, high affinity choline transport; p-choline, phosphocholine; CDP-choline, cytidine diphosphocholine; PC, phosphatidylcholine; Ach, acetylcholine; CK, choline kinase; ChAT, choline acetyltransferase; CT, CTP:phosphocholine cytidylyltransferase; CPT, cholinephosphotransferase.

CHAPTER 3

CHOLINE TAKEN UP BY THE CELL BODIES OF SYMPATHETIC NEURONS IS NOT UTILIZED FOR ACETYLCHOLINE SYNTHESIS

3.1 INTRODUCTION

Synthesis of Ach is believed to occur primarily in the axon terminals of cholinergic neurons. ChAT, the enzyme that catalyzes the synthesis of Ach, is synthesized within cell bodies, like other neuronal proteins, and transported down the axon to axon terminals (1). Histochemical and immunocytochemical studies have shown that ChAT is distributed throughout all parts (cell body, dendrites, axon and axon terminals) of cholinergic neurons (2, 3). In subcellular fractionation studies of brain homogenates, however, ChAT is enriched in synaptosomal fractions suggesting that the enzyme accumulates in nerve terminals (4, 5). Fonnum *et al.* (6) have estimated that of the total ChAT in cholinergic neurons of the hypoglossal nucleus, 2% is distributed in cell bodies, 42% in axons and 56% in axon terminals.

Choline utilized for Ach synthesis is taken up via a specialized HACT system (7-10). Denervation studies (11, 12) and studies examining choline uptake in synaptosomal preparations isolated by subcellular fractionation (7-10) suggest that HACT is localized to the axon terminals of cholinergic neurons. Some investigators have speculated that HACT and ChAT are physically (13) or kinetically coupled (14) since choline transported via HACT is rapidly acetylated. There is evidence, however, that Ach can be synthesized from an intrasynaptosomal pool of choline (15-17), as well as from choline immediately transported by HACT. Thus, whether or not choline transported via HACT mixes with intracellular pools of choline and whether Ach can be synthesized from these intracellular pools of choline has not been clearly defined.

The compartmented culture system of neurons (Fig. 3.1) is an excellent model in which processes occurring in axons and axon terminals can be studied independently of processes occurring in cell bodies (18, 19). The compartmented culture system was utilized in the following study in order to address the question of whether or not choline taken up by cell bodies can be utilized for Ach synthesis either locally within cell bodies or distally within axons and axon terminals. Additionally, the localization of HACT within rat sympathetic neurons was examined.

3.2 Materials and Methods

[*methyl*-^{3}H]Choline chloride (81 Ci/mmol) was obtained from NEN Products (Boston, MA). All other reagents and chemicals used are listed in Chapter 2 Section 2.2.

Preparation of Compartmented Cultures of Rat Sympathetic Neurons

A detailed description of the construction of compartmented culture dishes (Fig. 3.1) is described by Campenot (18, 19). Falcon tissue culture dishes (35 mm) are coated with rat tail collagen and scratched so that 20 parallel tracks are formed on the dish surface. A Teflon divider is sealed to the floor of the dish with silicone grease thus partitioning the dish into three compartments. Tracks are typically 200 μm wide, the center compartment 1 mm wide, the side compartments 4-5 mm wide and the barriers are about 0.5 mm wide (Fig. 3.1). After trypsinization and mechanical dissociation of

superior cervical ganglia, the dissociated neurons are plated in the center compartment (center-plated) of newly constructed compartmented dishes at a density of 0.6 to 0.8 ganglia/dish. Within 1-2 days axons elongate along the tracks, penetrate the silicone grease barrier beneath the divider and enter into the side compartments. After 1-2 weeks of growth, the center compartment contains the cell bodies and proximal neurites of the neurons and the side compartments contain the distal axons and axon terminals (Fig. 3.1). Each compartment maintains a separate fluid environment with virtually no flow between the compartments (20, 21). General procedures for the culture of rat sympathetic neurons were described in Chapter 2, Section 2.2. L15 medium without antibiotics, but supplemented with the additives prescribed by Hawrot and Patterson (22), including bicarbonate and methylcellulose, is used as the basal culture medium. For the first 6 days of growth, medium supplied to the center cell body-containing compartment is supplemented with 2.5% rat serum, 1 mg/ml ascorbic acid, 10 μM cytosine arabinoside (to prevent the growth of non-neuronal cells) and 10 ng/ml NGF. Medium supplied to the side compartments of center-plated cultures contains 100 ng/ml NGF. After 6 days, cytosine arabinoside and NGF treatment is discontinued in the center compartment and NGF is confined to the side compartments. Culture medium is routinely changed every 3-6 days.

Incorporation of [^{3}H]choline into Ach, PC and intermediates of PC biosynthesis

Compartmented neuron cultures were grown for 1 week and then treated with 5 μM RA in all three compartments for 9 days. Cultures were then given 10 μCi/ml

[*methyl*-^{3}H]choline in either the center or side compartments and incubated for 5 h or 24 h at 37°C in a 5% CO_2 incubator. The choline concentration in the medium was approximately 10 μM. Cells were washed and the cellular material from the center and side compartments was harvested separately and processed as described in Chapter 2, Section 2.2.

Choline Uptake Assay

Choline uptake assays were performed on 2.5 week old compartmented cultures or mass cultures of neurons. The center and side compartments of compartmented cultures were assayed in parallel. In control experiments, no transport of choline between compartments was detected during a 5 min labeling with [^{3}H]choline in either the center or side compartments alone. Assays were performed as described in Chapter 2 Section 2.2.

3.3 Results

3.3.1 Choline taken up by cell bodies is not incorporated into Ach

In order to determine if choline taken up by the cell bodies of cholinergic neurons is utilized for the synthesis of Ach, 1 week-old compartmented cultures of sympathetic neurons were treated for 9 days with 5 μM RA in all compartments. These conditions have previously been shown to induce cholinergic differentiation in rat sympathetic neurons (23, 24 and see Chapter 2). [^{3}H]Choline was supplied for 24 h to either the center, cell body-containing compartment alone or to the side, distal axon-containing

compartments alone. Cellular material from the center and side compartments was harvested separately and the incorporation of [^{3}H]choline into the metabolites of the CDP-choline pathway and into Ach was measured in each compartment. In cultures given [^{3}H]choline in the center compartment, 46% of the radiolabel taken up by the neurons was anterogradely transported into the side compartments (Fig. 3.2 bottom panel). Very little incorporation of [^{3}H]choline into Ach was observed in either the center or side compartments (Fig. 3.2 top and bottom panels). Most of the radiolabel (approximately 98%) was recovered in metabolites of the CDP-choline pathway, choline, phosphocholine, CDP-choline and PC (Fig. 3.2 top and bottom panels). Thus, although the cell bodies and proximal neurites of RA-treated sympathetic neurons readily take up [^{3}H]choline and a significant amount of radiolabel is anterogradely transported and present as [^{3}H]choline in both cell bodies and axons after 24 h, this [^{3}H]choline is poorly incorporated into Ach. In cultures supplied with [^{3}H]choline in the side compartments alone, 11% of the radiolabel in the side compartments was incorporated into Ach (Fig. 3.3 bottom panel). The remainder of the [^{3}H]choline was recovered as choline (13%), phosphocholine (37%), CDP-choline (0.5%) and PC (38%) (Fig. 3.3 bottom panel). Of the total radiolabel taken up by cultures given [^{3}H]choline to the side compartments, 16% of the radiolabel was retrogradely transported to the center compartment (Fig. 3.3 top panel). Most of the radiolabel transported into the center compartment (98%) was incorporated into products of the CDP-choline pathway (Fig. 3.3 top panel). Thus, the distal axons of RA-treated sympathetic neurons utilize locally supplied extracellular choline for both Ach and PC

synthesis. Similar results were obtained when RA-treated compartmented cultures were labeled with [^{3}H]choline in either the center or side compartments for only 5 h (Fig. 3.4 and 3.5). Under these conditions, when [^{3}H]choline was supplied to the center compartment, most of the radiolabel was incorporated into metabolites of the CDP-choline pathway (approximately 97%) in either the center or side compartments (Fig. 3.4). When [^{3}H]choline was supplied to the side compartments, however, 21.7% of the radiolabel in the side compartments was recovered in Ach and the remainder in choline (24%), phosphocholine (41%), CDP-choline (1%) and PC (13%). Radiolabel retrogradely transported back to the center compartment was predominantly in the intermediates of PC biosynthesis (91%) (Fig. 3.5). Thus, the same profile of [^{3}H]choline incorporation was observed under these conditions although less radiolabel was transported in 5 h and proportionately more radiolabel was recovered in the water soluble metabolites than in PC at 5 h versus 24 h. These experiments suggest that Ach is synthesized exclusively within the distal axons of sympathetic neurons and that extracellular choline is utilized for Ach synthesis.

3.3.2 HACT is localized to the distal axons of sympathetic neurons

The source of choline for Ach synthesis is believed to be extracellular choline transported into the neuron via HACT (7-10). One possible explanation for the restricted synthesis of Ach within distal axons of sympathetic neurons from locally supplied choline is that HACT is localized to the distal axons of sympathetic neurons. To test this hypothesis, choline uptake was measured in the center and side

compartments of compartmented cultures of sympathetic neurons. Non-RA-treated sympathetic neurons were used since it has been previously shown that HACT is present within cultures of sympathetic neurons and RA does not affect HACT (Chapter 2 Section 2.2.2). Center or side compartments were labeled for 5 min with [^{3}H]choline in the absence or presence of 10 μM HC-3, 50 μM HC-3 or in Na^+-free buffer. As seen in Fig. 3.6A, a small amount of choline was taken up by the cell bodies and proximal neurites in the center compartment (1.6 ± 0.8 nmol/5 min/mg protein) and this uptake was unaffected by HC-3 treatment or the absence of Na^+. Much more choline was taken by the distal axons in the side compartments (15.3 ± 4.4 nmol/5 min/mg protein) and this uptake was sensitive to inhibition by HC-3 and was dependent on external Na^+ (Fig. 3.6A). The characteristics of choline uptake seen in the side compartments of compartmented cultures of sympathetic neurons were similar to the profile of choline uptake seen in mass cultures (Fig. 3.6B and Chapter 2 Section 2.3.2). These results show that HACT is localized to the axons and axon terminals, but not to the cell bodies, of rat sympathetic neurons.

3.4 Discussion

The results from this study suggest that choline taken up by the cell bodies of RA-treated sympathetic neurons is not utilized for Ach synthesis either locally or distally in axons and axon terminals. These results further suggest that choline destined for Ach synthesis does not arise from an intracellular pool of choline but arises immediately from extracellular choline transport. Alternatively, two intracellular

pools of choline might exist within neurons which do not mix, one pool supplied by choline transported by HACT and utilized for Ach and PC synthesis and another pool supplied by low affinity transport used for PC synthesis exclusively.

Cell bodies and proximal neurites of sympathetic neurons readily take up [^{3}H]choline and incorporate the radiolabel into the intermediates of PC synthesis both locally and in distal axons (Fig. 3.2). The radiolabel, however, is not incorporated into Ach (Fig. 3.2). Incorporation of [^{3}H]choline into Ach was only observed when distal axons were supplied with [^{3}H]choline (Fig. 3.3). One issue which arises from these results is in what form the ^{3}H label is transported down the axon. The lack of incorporation of [^{3}H]choline, taken up by cell bodies, into Ach within distal axons can be explained if choline itself is not anterogradely transported. Choline taken up by cell bodies may be rapidly phosphorylated to phosphocholine and transported in this form which is not readily utilized for Ach synthesis. In cultures given [^{3}H]choline to the center compartments alone, however, the profile of distal radiolabel incorporation into choline, phosphocholine, CDP-choline and PC in the side compartments is similar to the profile of local radiolabel incorporation in the center compartment (Fig. 3.2 top and bottom panels). If phosphocholine or even CDP-choline or PC, were transported instead of choline, it is reasonable to assume that a different profile of radiolabel incorporation in the center and side compartments would be observed. Another possible explanation of these results is that the [^{3}H]choline taken up by cell bodies and transported into the axons might be diluted into a large pool of unlabeled choline.

Therefore, no incorporation of labeled choline into Ach would be observed. If this were true, however, the same effect should apply to the incorporation of [^{3}H]choline into PC in distal axons and no incorporation into the intermediates of the CDP-choline pathway would be observed. As seen in Fig. 3.2, however, [^{3}H]choline taken up by cell bodies was readily incorporated into the intermediates of the CDP-choline pathway and PC in distal axons.

HACT is localized to the axons and axon terminals, but not to the cell bodies, of rat sympathetic neurons (Fig. 3.6). An explanation of the [^{3}H]choline incorporation results in Fig. 3.2 and 3.3, therefore, is that choline utilized for Ach synthesis is taken up via a specialized HACT system localized to the axons and/or axon terminals of sympathetic neurons. In compartmented cultures of sympathetic neurons, Ach synthesis would, therefore, only occur in the side compartments which contain the axons and axon terminals. This has previously been suggested to occur in cholinergic neurons (7-10). HACT is believed to be localized to cholinergic nerve terminals since HACT activity is enriched in synaptosomal preparations isolated by subcellular fractionation (7-10) and loss of HACT has been observed in denervation experiments (11, 12). That HACT is an important aspect of Ach synthesis is supported by inhibition studies. Conditions that inhibit HACT, such as removal of extracellular Na^+ or treatment with HC-3 or choline mustard aziridinium ion, also affect Ach synthesis (8, 10, 25, 26). HACT is modulated by neuronal activity showing an increased rate of transport during periods of synaptic activity (27-30) and thus is thought to play a key

role in the synthesis and maintenance of Ach stores (31, 32). Experiments presented in this Chapter suggest that choline taken up by HACT does not mix with intracellular pools of choline. Choline from HACT might be utilized very quickly after transport for the synthesis of Ach or phosphocholine such that accumulation of choline does not occur. Alternatively, separate compartments or pools of choline originating from HACT or low affinity transport might exist which do not mix.

These results do not rule out the possibility that if the extracellular supply of choline is limiting, intracellular choline or choline mobilized from the action of phospholipases on PC is utilized for Ach synthesis (33). Repeated depolarization of striatal slices (33, 34) causes a decrease in membrane PC which is rescued by choline administration (34). Evidence for Ach synthesis from choline derived from PC in neuronal cell lines has been provided (35, 36).

The results from this Chapter indicate that choline used for Ach synthesis arises from extracellular choline taken up locally by the axons and axon terminals of RA-treated rat sympathetic neurons. The results further suggest that intracellular choline is not a source of choline for Ach synthesis. Choline taken up by cell bodies was not incorporated into Ach within the cell bodies themselves or distally within the axons and axon terminals.

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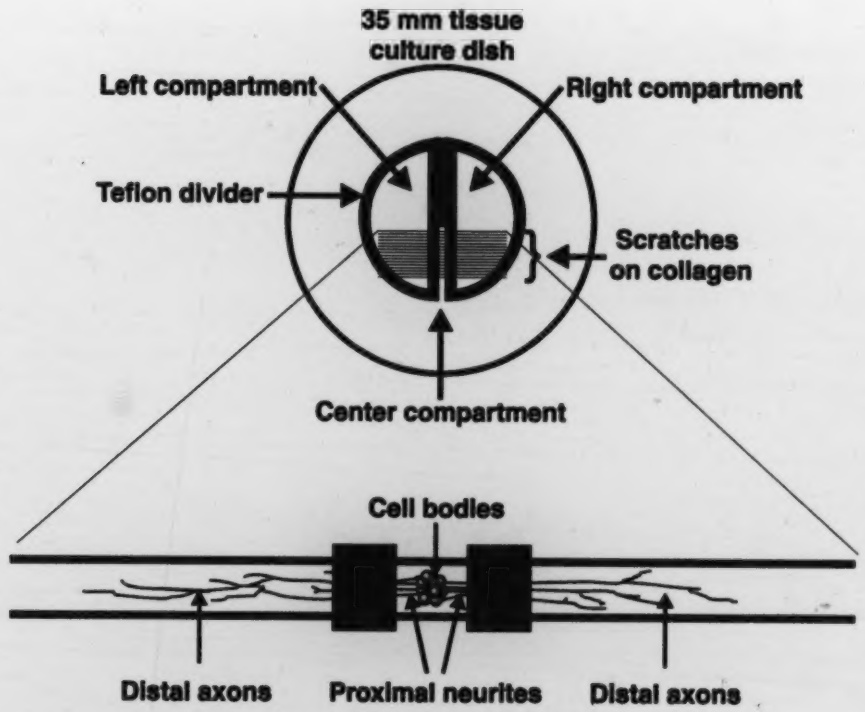
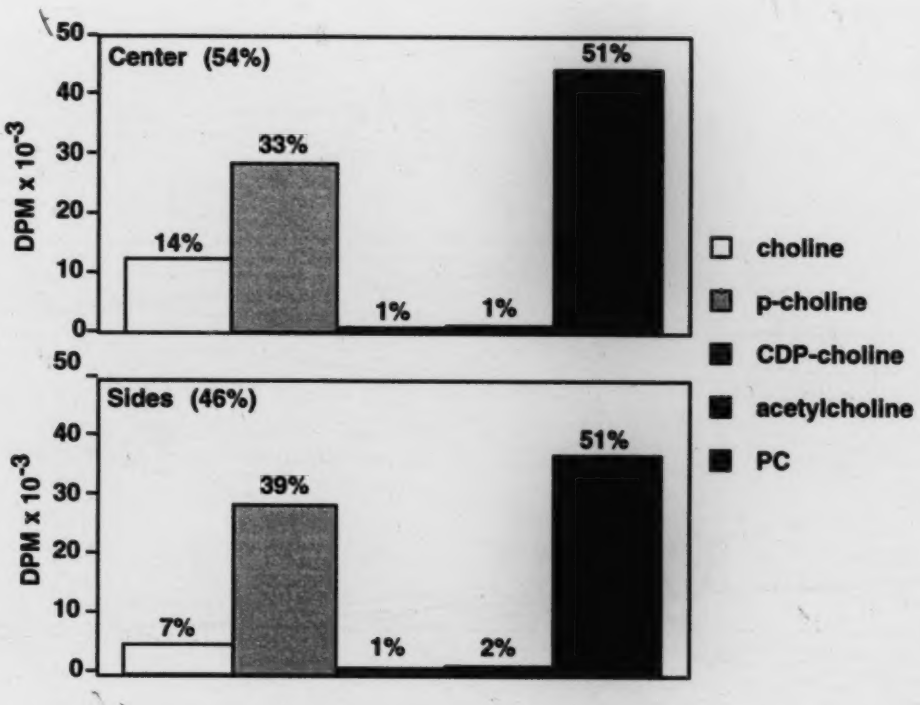


Fig. 3.1 **Schematic diagram of a compartmented culture**

A compartmented culture and the enlargement of a single track are illustrated. Collagen-coated 35 mm Falcon tissue culture dishes are scratched so that 20 parallel tracks are formed on the dish surface. A Teflon divider is sealed to the floor of the dish with silicone grease thus partitioning the dish into three compartments. Tracks are typically 200 μm wide, the center compartment 1 mm wide and the barriers are about 0.5 mm wide. Cell bodies isolated from enzymatically and mechanically dissociated ganglia are plated in the center compartment (density 0.6-0.8 ganglia per dish). Within 1-2 days neurites elongate along the tracks, penetrate the silicone grease barrier beneath the teflon divider and enter the left and right compartments.

Fig. 3.2 **Choline taken up by cell bodies is not incorporated into Ach.**

Compartmented cultures of sympathetic neurons were treated with 5 μM retinoic acid for 9 days prior to the start of the experiment. The center compartments were incubated for 24 h with medium containing 10 μCi/ml [*methyl*-^{3}H]choline. The radiolabel incorporated into choline, phosphocholine (p-choline), CDP-choline, Ach (acetylcholine) and PC (phosphatidylcholine) in the center (top panel) and side (bottom panel) compartments was measured. The percentage in parentheses at the top of each panel indicates the percentage of the total label taken up which was incorporated in the center (top panel) or side (bottom panel) compartments. Percentages above the bars indicate the percentage of radiolabel in the compartment incorporated into the product indicated by the bar. Shown is the sum of the incorporation into 3 dishes. The experiment was done in triplicate with a similar profile of radiolabel incorporation observed in each replicate. The experiment was repeated once with similar results.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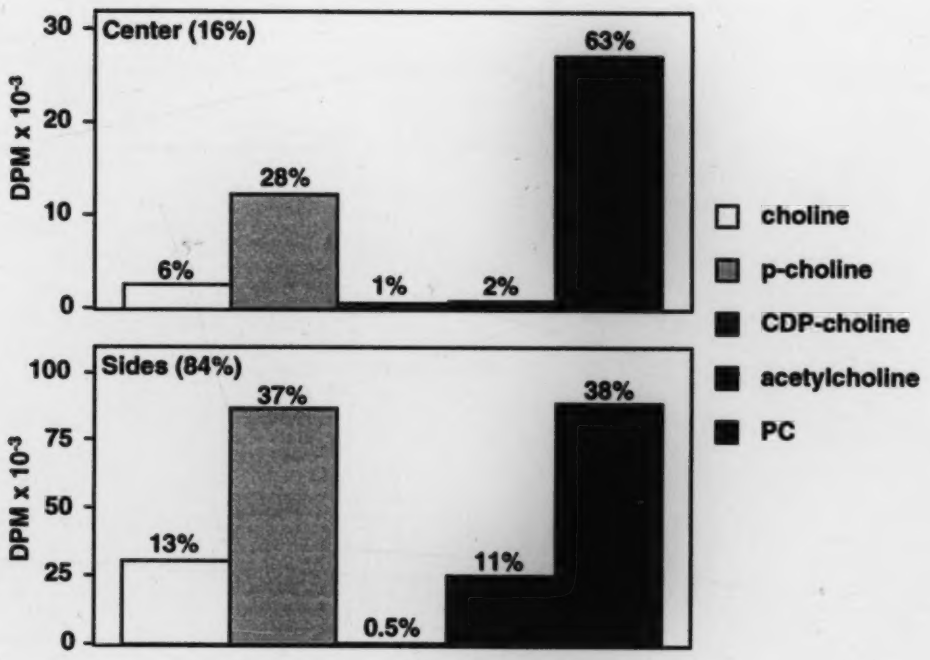
CDP-choline
acetylcholine
PC

Fig. 3.3 [^{3}H]choline is incorporated into Ach in axons and axon terminals of rat sympathetic neurons

Compartmented cultures of sympathetic neurons were treated with 5 μM retinoic acid for 9 days prior to the start of the experiment. The side compartments were incubated for 24 h with medium containing 10 μCi/ml [*methyl*-^{3}H]choline. The radiolabel incorporated into choline, phosphocholine (p-choline), CDP-choline, Ach (acetylcholine) and PC (phosphatidylcholine) in the center (top panel) and side (bottom panel) compartments was measured. The percentage in parentheses at the top of each panel indicates the percentage of the total label taken up which was incorporated in the center (top panel) or side (bottom panel) compartments. Percentages above the bars indicate the percentage of radiolabel in the compartment incorporated into the product indicated by the bar. Shown is the sum of the incorporation into 3 dishes. The experiment was done in triplicate with a similar profile of radiolabel incorporation observed in each replicate. The experiment was repeated once with similar results.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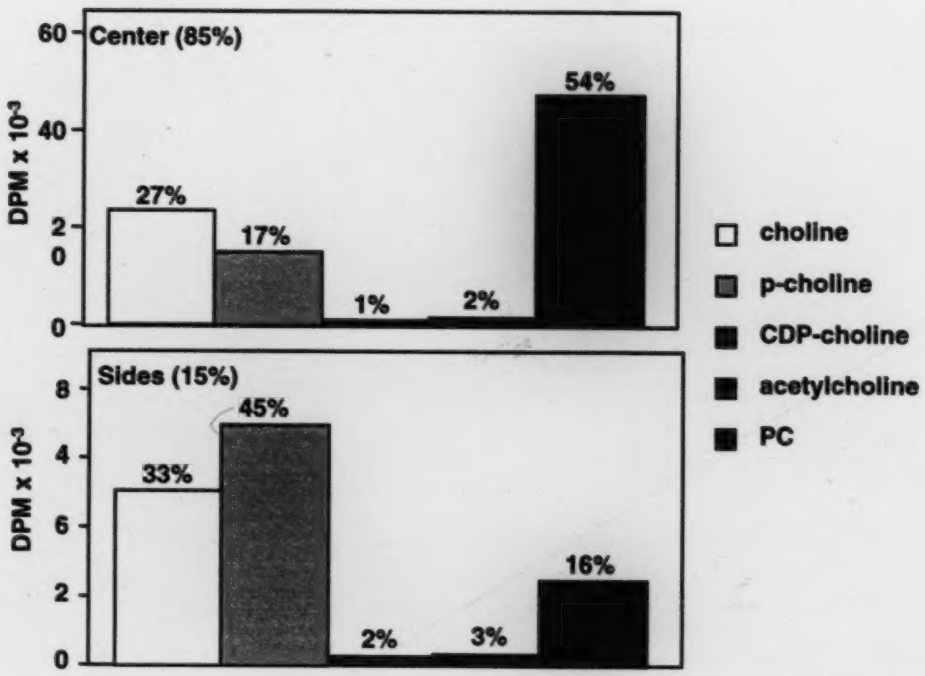
acetylcholine
PC

Fig. 3.4 **Choline taken up by cell bodies is not utilized for Ach synthesis**

See the legend of Fig. 3.2 for experimental details. Cultures were radiolabeled in the center compartment with [*methyl*-^{3}H]choline for 5 h. The radiolabel incorporated into choline, phosphocholine (p-choline), CDP-choline, Ach (acetylcholine) and PC (phosphatidylcholine) in the center (top panel) and side (bottom panel) compartments was measured. The percentage in parentheses at the top of each panel indicates the percentage of the total label taken up which was incorporated in the center (top panel) or side (bottom panel) compartments. Percentages above the bars indicate the percentage of radiolabel in the compartment incorporated into the product indicated by the bar. Shown is the sum of the incorporation into 3 dishes. The experiment was done in triplicate with a similar profile of radiolabel incorporation observed in each replicate. The experiment was repeated once with similar results.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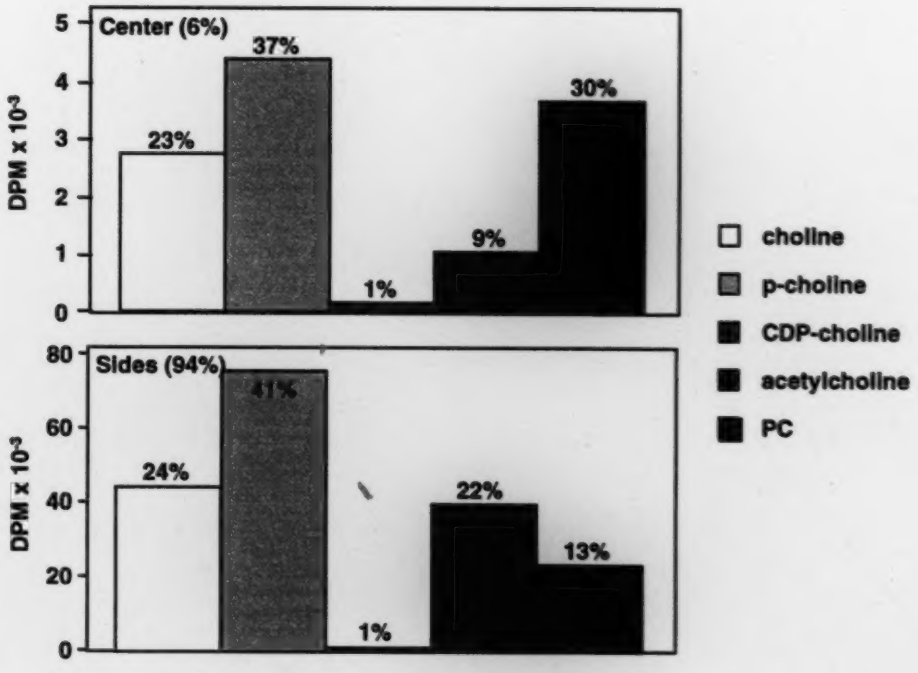
choline
p-choline
CDP-choline
acetylcholine
PC

Fig. 3.5 [^{3}H]choline is rapidly incorporated into Ach in axons and axon terminals

See the legend of Fig. 3.3 for experimental details. Cultures were radiolabeled in the side compartments with [*methyl*-^{3}H]choline for 5 h. The radiolabel incorporated into choline, phosphocholine (p-choline), CDP-choline, Ach (acetylcholine) and PC (phosphatidylcholine) in the center (top panel) and side (bottom panel) compartments was measured. The percentage in parentheses at the top of each panel indicates the percentage of the total label taken up which was incorporated in the center (top panel) or side (bottom panel) compartments. Percentages above the bars indicate the percentage of radiolabel in the compartment incorporated into the product indicated by the bar. Shown is the sum of the incorporation into 3 dishes. The experiment was done in triplicate with a similar profile of radiolabel incorporation observed in each replicate. The experiment was repeated once with similar results.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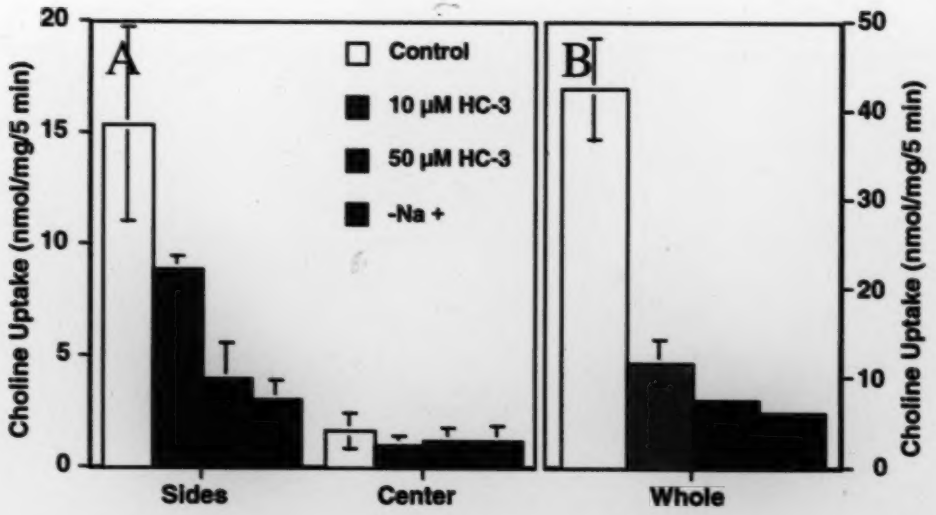

Fig. 3.6 **HACT is localized to the axons and axon terminals of cultured rat sympathetic neurons**

Compartmented cultures (panel A) or mass cultures (panel B) of sympathetic neurons were incubated for 5 min in Krebs-Ringer buffer containing 10 µCi/ml [*methyl*-^{3}H]choline (0.125 µM). The total radioactivity incorporated into the center and side compartments of compartmented cultures (panel A) or into mass cultures (panel B). Choline uptake in the presence of 10 µM HC-3, 50 µM HC-3 or in Na^+-free buffer was determined in parallel. Each value represents the mean ± S.D. of 4 mass culture dishes or compartmented culture dishes. The experiment was repeated twice with similar results.

CHAPTER 4

INTRODUCTION TO SPHINGOLIPIDS AND GLYCOSPHINGOLIPIDS

4.1 Introduction to Sphingolipids

In a book entitled *A Treatise on the Chemical Composition of the Brain* published in 1884, Johann Thudichum first described the existence of sphingolipids (1). Thudichum found that the hydrolysis of novel compounds discovered in the course of his analyses, such as sphingomyelin, cerebroside and cerebrosulfatide, lead to the formation of a substance which he called *sphingosin*, meaning "sphinx-like", due to its many enigmatic properties. These lipids were, therefore, named the *sphingolipids* in reference to their common sphingosine backbone. Although, sphingolipids are ubiquitous constituents of membranes in eukaryotes and prokaryotes, the first sphingolipids described, such as sphingomyelin and cerebrosides, were named from the tissue from which they were originally isolated. More than 300 different types of sphingolipids have been described highlighting the enormous diversity present within this class of lipids.

Sphingolipids are defined by their unique structure (Fig. 4.1). All sphingolipids contain a long chain sphingoid base backbone. The most common sphingoid base in mammalian tissues is sphingosine. Phytosphingosine (4-hydroxysphinganine) is the most common backbone in yeast and plants but exists only in small percentages in mammalian sphingolipids. Attached to this backbone is an amide linked fatty acyl group. This fatty acyl group is typically unbranched, saturated and 16 to 24 carbons in length. Sphingolipids are classified into different groups depending on the head group located at position 1 of the backbone. The headgroup of glycosphingolipids (GSLs) is

a hydrophilic carbohydrate chain containing as few as one to as many as 30 or more sugar residues attached via a glycosidic linkage to carbon-1 of the backbone. Neutral GSLs contain non-polar sugars, such as glucose, galactose, *N*-acetylglucosamine or fucose within their headgroup. The headgroup of acidic GSLs also contains charged groups such as phosphate, sulfate or sialic acid (*N*-acetylneuraminic acid). GSLs which contain sialic acid residues are referred to as gangliosides, a group of GSLs particularly enriched in neural tissue. Sphingomyelin (SM) is a sphingolipid which contains a phosphocholine group bound to carbon-1 of the sphingoid base and can be classified as a phosphosphingolipid. Variation in the headgroup, in addition to variation in the length, branching and degree of unsaturation of the sphingoid base backbone and the amide-linked fatty acyl chain, contributes to the heterogeneity observed within the sphingolipids.

Significant interest in the sphingolipids was generated by the discoveries that elevated levels of sphingolipids are present in the tissues of patients afflicted by a variety of genetic diseases such as Niemann-Pick, Tay-Sachs and Gauchers disease (reviewed in 2). These discoveries generated intense research aimed at elucidating the genetic defects causing these disease phenotypes and the cloning and characterization of the proteins involved. More recently, renewed interest in this class of lipids has come with the findings that sphingolipids play a role in a number of important cellular processes including cell-cell and cell-substratum adhesion, differentiation, proliferation and oncogenic transformation (3-5). In addition, intermediates in the

biosynthesis and catabolism of sphingolipids and GSLs may also function as lipid second messengers mediating the effects of extracellular agents and agonists (6-8).

4.2 Synthesis of sphingolipids

4.2.1 Synthesis of ceramide

The initial steps in sphingolipid synthesis lead to the production of ceramide, a branch point in the synthesis of more complex sphingolipids (Fig. 4.2). Ceramide is a central molecule of sphingolipid metabolism being both a key intermediate in the synthesis of sphingolipids and a product of their catabolism (see Section 4.3 and Fig. 4.2 and 4.5). Ceramide is formed from the condensation of palmitoyl-CoA and serine producing 3-ketosphinganine which is rapidly reduced to sphinganine. Sphinganine is acylated to generate dihydroceramide which is then desaturated to generate ceramide (Fig. 4.2). The enzymatic reactions leading to the production of ceramide have been localized to the cytosolic side of the endoplasmic reticulum. The subsequent glycosylations and modifications of ceramide to generate the complex sphingolipids occur within the lumen of the Golgi apparatus. Although important advances have been made in defining the biochemical pathway used by cells to generate sphingolipids, little is known about the regulation of sphingolipid synthesis. Progress in this area has been slow since few of the enzymes which are involved in the synthesis of sphingolipids have been purified or characterized at the molecular level.

Serine palmitoyltransferase (SPT)

The synthesis of all sphingolipids is initiated by the condensation of palmitoyl-CoA and L-serine to generate the long chain base 3-ketosphinganine (Fig. 4.2). This reaction, first demonstrated *in vitro* by Brady and Koval (9), is catalyzed by the enzyme serine palmitoyltransferase (SPT) (Fig. 4.2) (10-12). SPT requires pyridoxal 5'-phosphate as a cofactor (9, 12) and has been localized to the cytosolic face of the endoplasmic reticulum (13). The enzyme is highly specific for linear saturated fatty acyl-CoAs of 16 ± 2 carbon atoms (12), which explains the prevalence of 18 carbon sphingoid bases (2 carbons derived from serine and 16 carbons from palmitoyl-CoA) in most sphingolipids (14). Yeast mutants defective in SPT activity have been isolated, called lcb (long chain base) mutants, which are sphingoid base auxotrophs (15, 16). The yeast SPT was cloned by genetic complementation of these mutants (15, 17).

Evidence suggests that the first step of sphingolipid synthesis is also the rate-limiting and regulated step (18, 19). SPT catalyzes the first committed step of sphingolipid synthesis and the activity of this enzyme has been determined to be lower than the activity of the subsequent enzyme in the pathway, 3-ketosphinganine reductase (20). In accordance with this observation, 3-ketosphinganine is not detected in intact cells indicating its rapid reduction to sphinganine (21). The enzyme activity of SPT correlates with the content of sphingolipids in various tissues (20) and is increased under conditions where sphingolipid synthesis is increased, such as in regenerating rat

liver (22). Further evidence that the activity of SPT controls the flux through this pathway is provided by inhibitor studies. Several inhibitors of SPT have been discovered, such as cycloserine, β-fluoroalanine and myriocin, which cause a decrease in long-chain base synthesis parallel to their inhibition of SPT activity (23, 24). Similarly, due to the dependence of SPT on pyridoxal 5'-phosphate, vitamin B_6-deficiency reduces the incorporation of [^{3}H]serine into sphingolipids in developing rat brain (25). SPT is subject to feedback regulation by the long chain base sphingosine (26, 27), a catabolic product of sphingolipids, perhaps by transcriptional downregulation. SPT activity was reduced by 50% in cerebellar neurons cultured in the presence of 25 μM sphingosine for 4 h (26, 27). These observations suggest that SPT may be a regulatory point within the sphingolipid biosynthetic pathway.

3-Ketosphinganine reductase catalyzes the next step in sphingoid base synthesis, the reduction of 3-ketosphinganine to sphinganine (28) (Fig. 4.2). This reaction is NADPH-dependent and, as previously mentioned, appears to be very active *in vivo* since 3-ketosphinganine does not accumulate and is not detected in cells (20, 21). This enzyme activity has also been localized to the cytosolic face of the endoplasmic reticulum (13).

Sphinganine *N*-acyltransferase

Formation of dihydroceramide occurs upon fatty acylation of the amino group of sphinganine (Fig 4.2) primarily via a fatty acyl-CoA-dependent pathway (21, 29, 30).

CoA-independent synthesis of dihydroceramide has been described, however (31, 32). Acylation of sphinganine occurs very rapidly *in vivo* since free sphinganine is not detected as an intermediate in sphingolipid synthesis (21). Free sphingoid bases have potent biological effects on cells (33) and can be cytotoxic (34). Thus, the rapid acylation of sphinganine to dihydroceramide might be advantageous for a cell in terms of survival (18). The low amounts of free sphingoid bases found in cells are thought to arise from the turnover of sphingolipids (18). Similar to the first two steps in sphingolipid synthesis, acylation of sphinganine has been shown to occur on the cytosolic face of the endoplasmic reticulum (13, 35).

Fumonisin B_1 (FB_1), a compound produced by *Fumonisarium moniliforme*, one of the most common fungal contaminants of agricultural products such as corn and other grains, is a potent naturally occurring inhibitor of sphinganine *N*-acyltransferase (36 and see Chapter 5). FB_1 is structurally very similar to sphinganine and is thought to occupy both the fatty acyl-CoA and sphinganine binding sites of sphinganine *N*-acyltransferase and thus inhibit dihydroceramide synthesis (37, 38). Inhibition of this enzyme leads to an accumulation of free sphinganine and a reduction in total sphingolipids in cells which has been correlated with the growth inhibitory and cytotoxic effects of FB_1 on cells (39, 40).

Dihydroceramide desaturase

The introduction of the 4,5-*trans* double bond of sphingolipids has been proposed (21, 41, 42) and recently proven (43) to occur subsequent to the production of dihydroceramide (Fig. 4.2). Free sphingosine is, therefore, not an intermediate in the *de novo* biosynthesis of sphingolipids, but is a catabolic product of ceramide (see Section 4.3 and Fig. 4.5). The subcellular localization of dihydroceramide desaturase activity has not been determined but this reaction has been postulated to take place in a different subcellular compartment from dihydroceramide synthesis (21).

4.2.2 Synthesis of sphingomyelin

One of the metabolic fates of ceramide is the formation of sphingomyelin (Fig 4.2). The mechanism and site of synthesis of SM, as well as its intracellular transport, have been the subject of numerous investigations and reviews (18, 44-46). SM is predominantly localized to the outer leaflet of the plasma membrane, and related membranes such as endocytic vesicles and lysosomes of cells and is also found in lipoproteins (18, 44, 46). It accounts for approximately 5% of the total phospholipid in liver (18) and up to 25% of the phospholipid of lipoproteins (47). Interestingly, a correlation between the amount of SM and the amount of cholesterol in different membranes has been observed (48). This observation, in addition to studies showing a close association between the metabolism of SM and cholesterol (49, 50), has led to the speculation that SM might play an important role in cholesterol metabolism and mobilization. A role for SM in cell signaling has recently been described with the

discovery of a new signal transduction pathway initiated by the hydrolysis of SM (7, 8). Numerous cytokines and environmental stressors induce SM hydrolysis generating the lipid second messenger ceramide which is believed to mediate, at least in part, the effects of these extracellular agents on cells (7, 8, also see Section 4.4 below).

PC:ceramide cholinephosphotransferase or sphingomyelin synthase

The *in vitro* synthesis of SM was first demonstrated by Sribney and Kennedy (51) by incubation of ceramide and CDP-choline in a cell-free system. These investigators also noticed that the *threo*-isomer of ceramide was a much better substrate than the *erythro*-isomer of ceramide for the formation of SM (51). Although these initial studies demonstrated that CDP-choline could function as a phosphocholine donor in the synthesis of SM *in vitro*, subsequent studies have suggested that PC is a more likely donor. This idea was first suggested from [^{3}H]choline and $^{32}P_i$ pulse-chase studies when it was observed that radiolabel could be chased from PC into SM (52). Subsequent analyses of SM formation (52-54) have supported this hypothesis. Marggraf and Anderer (55) have estimated that synthesis of SM from CDP-choline accounts for only 2% of SM synthesis in mouse fibroblasts.

The subcellular localization of SM synthesis has been the subject of much debate but the major site of synthesis is now believed to be the Golgi apparatus (18, 45, 56). Early subcellular fractionation studies showed that SM synthase activity localized primarily to the plasma membrane but also to Golgi, mitochondria and microsomal

fractions (53, 57). Observations from experiments using fluorescent analogs of ceramide suggested that the Golgi was the major site of SM synthesis (58, 59). Fluorescence from cells incubated with NBD-ceramide (*N*-[7-(4-nitrobenxo-2-oxa-1,3-diazole]aminocaproylsphingosine) analogs, was quickly associated with regions of the cell identified as Golgi apparatus and was recovered in NBD-SM (58). Longer incubations resulted in the accumulation of fluorescence at the plasma membrane. Inhibition of vesicular transport from the Golgi apparatus to the plasma membrane using the drug monensin decreased the fluorescence recovered at the plasma membrane without affecting NBD-SM synthesis (58). These results suggest that NBD-ceramide initially localizes to the Golgi apparatus, is converted to NBD-SM and is subsequently transported to the PM. Additional evidence for the localization of SM synthesis to Golgi membranes was provided by careful separation and characterization of subcellular fractions (60, 61). Thus, it is generally agreed that approximately 90% of SM synthesis occurs in the Golgi apparatus and 10% at the plasma membrane (56, 62)

SM synthase has been solubilized and reconstituted from Chinese hamster ovary cells (63). In this preparation, reconstitution of full activity required PC and the enzyme activity was inhibited by diacylglycerol (63), suggesting that the enzyme might be regulated by feedback inhibition. Although many factors such as phorbol esters (64), glucocorticoids (65) and vitamin D_3 (66), are known to stimulate SM synthesis, it is not known at what level these compounds affect SM synthesis. Possible sites of

regulation include serine palmitoyltransferase, SM synthase and sphingomyelinases, which catalyze the breakdown of SM (see Section 4.3).

4.2.3 Synthesis of glycosphingolipids

GSLs are a diverse group of amphiphilic molecules predominantly located in the outer leaflet of the plasma membrane of cells (67). GSLs are oriented in the plasma membrane such that the lipophilic ceramide portion of the molecule is embedded in the membrane and the hydrophilic carbohydrate chain protrudes into the extracellular milieu. Gangliosides are a special group of GSLs which contain sialic acid and are highly enriched in neuronal plasma membranes (19, 68).

Assembly of the oligosaccharide moiety of GSLs occurs predominantly within the Golgi apparatus en route to the plasma membrane (19, 69). Synthesis occurs by the stepwise transfer of a single sugar, from an appropriate sugar nucleotide, to the non-reducing end of the growing carbohydrate chain attached to ceramide. Each step is catalyzed by a specific glycosyltransferase (69-71). Since the assembly of the oligosaccharide chain occurs in a strictly ordered sequence, GSLs can be classified into families based on the sequence of their neutral oligosaccharide core which is indicative of a common route of biosynthesis (Fig. 4.3). It is obvious that numerous glycosyltransferases are required to account for the diversity of naturally occurring GSLs.

Glucosylceramide synthase catalyzes the transfer of glucose from UDP-glucose to ceramide to generate glucosylceramide. This is the initial step in the assembly of the oligosaccharide moiety of most GSLs (Fig. 4.2). The exact location of glucosylceramide synthesis has not been determined. Incubation of cultured cells with fluorescent analogs of ceramide demonstrated that glucosylceramide was synthesized before it left the Golgi apparatus (58, 72). Subcellular fractionation studies suggest that glucosylceramide is synthesized on the cytosolic surface of Golgi membranes (73-76) and in a pre-Golgi-compartment (74, 75, 77). The nature of this second compartment has yet to be determined. Galactosyltransferase I, which catalyzes the synthesis of lactosylceramide (galactosylglucosylceramide), also appears to be localized to the cytosolic surface of Golgi membranes (75), although some studies have shown a luminal topology (78). It is not known how glucosylceramide or lactosylceramide traverse the Golgi membrane. The glycosyltransferases which catalyze the synthesis of more complex GSLs are membrane-bound and restricted to lumen of the Golgi apparatus (75, 79-81). A mechanism must, therefore, exist for the transbilayer movement or "flip-flop" of glucosylceramide and lactosylceramide from the cytosolic to the luminal side of Golgi membranes. A portion of the glucosylceramide synthesized by a cell bypasses further glycosylation and is transported directly to the plasma membrane without entering the Golgi apparatus (82). The mechanism for transport of glucosylceramide directly to the plasma membrane is unknown but might involve a cytosolic carrier protein (83).

Glucosylceramide synthase activity has been solubilized and reconstituted from Golgi membranes isolated from porcine submaxillary glands (84, 85). Full enzyme activity required a divalent cation, such as manganese, and was highly dependent on the lipid environment. Paul *et al.* (86) have recently reported a greater than 10,000-fold purification of glucosylceramide synthase from rat liver Golgi membranes. Two polypeptides of 60-70 kDa co-purified with glucosylceramide synthase activity. The purified enzyme required PC for full activity, preferentially used UDP-glucose as a hexose donor and was stereospecific, utilizing *erythro-* but not *threo-* ceramide as a substrate. This stereospecificity has previously been reported in cultured fibroblasts (87).

Potent inhibitors of glucosylceramide synthase have been developed by Radin and colleagues (88). PDMP (1-phenyl-2-decanoylamino-3-morpholino-1-propanol) and PPMP (1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) are structural analogs of ceramide and glucosylceramide and have been demonstrated to inhibit glucosylceramide synthesis in intact cells (89, 90 and see Chapter 5) and *in vitro* (86, 91). Blocking sphingolipid synthesis at this step results in the depletion of glycosphingolipids and gangliosides from cells and an increase in ceramide levels (89, 90).

Gangliosides of the ganglio family (Fig. 4.3) are the most abundant gangliosides in nervous tissue and are the most characterized and extensively studied group of GSLs.

The nomenclature for gangliosides was suggested by Svennerholm based on their carbohydrate structure (92). Thus, members of the ganglio family are designated by a capital G and are defined by the neutral sugar sequence galactose-N-acetylgalactosamine-galactose-glucose-ceramide (Fig. 4.3). The sialic acid content is designated by a subscripted capital letter: A for asialo-, M for monosialo-, D for disialo-, T for trisialo- and so forth, defining gangliosides containing zero to three sialic acid residues, respectively. The length of the neutral sugar chain is denoted by a subscripted number corresponding to the formula 5 - x, where x is the number of neutral sugars in the ganglioside. The larger the number, the shorter the chain. In addition, the position of the sialic acid residues can be further denoted by a subscripted lower case a, b or c. Using this nomenclature the ganglioside G_{M3} would therefore have the structure N-acetylneuraminic acid-galactose-glucose-ceramide.

The biosynthetic pathway for the synthesis of the ganglio type gangliosides was elucidated largely from the work of Basu and colleagues (reviewed in 71) and is depicted in Fig. 4.4. It has been found that most of the glycosyltranferases involved in the synthesis of gangliosides are rather unspecific for their acceptors (93-95). This observation explains the conservative number of glycosyltransferases required to synthesize the wide range of gangliosides. The localization of the glycosyltransferases within the Golgi stacks corresponds to the sequence of assembly of the oligosaccharide chain. Thus, simple gangliosides, such as G_{M3} and G_{D3} are synthesized in early Golgi compartments and more complex gangliosides, such as

G_{T1b} and G_{Q1b}, are synthesized in late Golgi compartments. This information was obtained from subcellular fractionation studies (96, 97) and metabolic studies using drugs which interfere with vesicular transport within the Golgi apparatus, such as monensin (98, 99) and Brefeldin A (100, 101). When cells were treated with these compounds, a decreased incorporation of radiolabel into complex gangliosides such as G_{Q1b}, G_{T1b}, G_{D1b}, G_{D1a} and G_{M1} and an accumulation of radiolabel in glucosylceramide, lactosylceramide, G_{M3} and G_{D3} was observed (98-101). Synthesis of gangliosides beyond G_{M3} and G_{D3} is, therefore, thought to occur in a late or *trans*-Golgi compartment. Further support for this hypothesis is provided by recent studies in which cells have been transfected with the cDNA for enzymes of this pathway (102, 103). It is important to recognize that GSLs and gangliosides of all stages of synthesis appear on the plasma membrane of cells in varying concentrations and therefore sphingolipid glycosylation does not always proceed to completion.

Little is known about the regulation of GSL and ganglioside biosynthesis. Changes in the pattern of GSLs and gangliosides expressed on cells during development, differentiation and oncogenic transformation have been observed, as well as differences in the composition of mammalian organs (3-5). These changes or differences are thought to be regulated at the transcriptional level such that the appearance of a GSL or ganglioside is mediated by the expression of the appropriate glycosyltransferase (19, 70). In agreement with this hypothesis, transfection of cells with the cDNA for specific glycosyltransferases increases the expression of the

respective GSLs or gangliosides (102, 103). There is some evidence that certain glycosyltransferases might be regulated by protein phosphorylation/dephosphorylation (104, 105). Another factor which can influence the synthesis of GSLs is the availability of sugar nucleotides (106). Sugar nucleotides are transported into the Golgi apparatus by specific transport proteins in the Golgi membrane which might regulate the availability of sugar nucleotides for GSL synthesis (106).

4.3 Degradation of Sphingolipids

Sphingolipid turnover in cells occurs in several ways including secretion, shedding, endocytosis followed by degradation or recycling and by hydrolysis, releasing bioactive lipids which participate in cell signaling (56). The intracellular degradation of sphingolipids has received intense research interest due to the discovery of diseases with functional defects in lysosomal sphingolipid catabolism (2, 56). More recently, the discovery that novel signal transduction pathways are initiated by sphingolipid degradation (7, 8) has prompted a new wave of interest in sphingolipids and sphingolipid degradation. Complex sphingolipids (GSLs, gangliosides, SM) are degraded to ceramide which can be recycled to the Golgi apparatus for sphingolipid synthesis or broken down completely.

4.3.1 Glycosidases

The oligosaccharide chain of GSLs is degraded in the lysosomes of cells via the stepwise hydrolysis of the terminal sugar residue by the concerted action of specific

acid exoglycosidases (Fig. 4.5) (107). Degradation of short GSLs requires the assistance of cofactors called SAPs (or saposins) for sphingolipid activator proteins (107, 108). SAPs bind to membrane-bound GSLs, lift them from the plane of the membrane and present them to the lysosomal hydrolases (107, 108). Lysosomal lipid storage diseases characterized by inherited functional deficiencies in many of these exoglycosidases or in SAPs have been described (2, 107). The severity of these diseases depends on the amount of residual enzyme activity since lipid accumulation dramatically increases below a critical threshold of enzyme activity (2, 107). Two examples of sphingolipid storage diseases are Tay Sachs and Gauchers disease. Tay Sachs disease is caused by a deficiency in β-*N*-acetylhexosaminidase activity due to mutations in the α subunit of the enzyme (2). A consequence of this deficiency is the accumulation of ganglioside G_{M2} since the terminal *N*-acetylgalactosamine residue of G_{M2} can no longer be removed. Classical Tay-Sachs is characterized by a rapidly progressing neurological degeneration. A murine model of Tay Sachs disease has recently been generated by targeted disruption of the gene coding for the α subunit of β-*N*-acetylhexosaminidase (109). Gaucher disease is caused by a deficiency in β-D-glucocerebrosidase which results in the accumulation of glucosylceramide due to a compromised ability to remove the glucose residue from glucosylceramide (2). Lipid accumulation in this disease is restricted to monocytes and macrophages. Both the cDNA and the gene of β-D-glucocerebrosidase have been cloned (110, 111). Gaucher disease was the first lysosomal storage disease treated successfully by enzyme replacement therapy (2).

4.3.2 Sphingomyelinases

SM is hydrolyzed to phosphocholine and ceramide by the action of sphingomyelinases (SMases) (Fig. 4.5). A number of SMases with different pH optima and divalent cation requirement have been characterized. The lysosomal hydrolysis of SM is catalyzed by an acid SMase (112, 113) which has been purified and the cDNA cloned (114). This enzyme is ubiquitously expressed, acts optimally at low pH (pH 4-5) and does not exhibit any dependency on divalent cations (115). Many cells secrete a Zn^{2+}-stimulated SMase, first detected in human serum (116), which has recently been demonstrated to arise from the acid SMase gene via a post-transcriptional process (117). Niemann-Pick disease (Type A and B) is characterized by the lysosomal accumulation of SM in cells of various tissues of the body due to a loss of acid SMase activity (115). A mouse model of Niemann-Pick disease (Type A) has recently been generated by genetic ablation of the acid SMase gene (118, 119).

Early studies by Schneider and Kennedy (120), described the existence of a neutral membrane-associated Mg^{2+}-dependent SMase. This enzyme has been localized to the plasma membrane of cells (121-123) with its active site oriented to the extracellular side of the membrane (122). Mg^{2+}-stimulated SMase activity is enriched in brain and kidney (124) and arises from a separate gene than lysosomal SMase since activity is normal in patients with Niemann-Pick disease types A and B (45) and in mice deficient in acid SMase activity (118, 119). Mg^{2+}-stimulated SMase has been partially

purified from various human tissues and urine (125). A neutral SMase localized to the cytosol of human myelocytic leukemia HL-60 cells has also recently been partially purified (126). This cytosolic neutral SMase is not dependent on divalent cations and might be activated by arachidonate (127).

4.3.3 Ceramidases

Ceramide produced by the hydrolysis of GSLs or SM is cleaved by the action of a ceramidase to produce sphingosine and a fatty acid (Fig. 4.5). Several ceramidases have been described with different pH optima. A lysosomal enzyme with an acidic pH optima (pH 4-5) has been purified from human urine (128) and the cDNA cloned (129). A functional defect in acid ceramidase activity leads to the disorder known as Farbers disease which is characterized by the lysosomal accumulation of ceramide in most tissues (130). A neutral ceramidase activity (optimum pH 7.6) (131) and a ceramidase with an alkaline pH optima (pH 9.0) have also been described (121, 132), although these enzymes have not been extensively characterized. Inhibitors of ceramidase activity have been developed and used in the investigation of sphingolipid metabolism and function: *N*-oleoyl-ethanolamine (132) and D-myristoylamino-phenyl-propanol (133). D-myristoylamino-phenyl-propanol inhibits alkaline, but not acid, ceramidase and results in the accumulation of ceramide (133).

4.3.4 Long chain base degradation

Sphingosine produced by the deacylation of ceramide is either phosphorylated by sphingosine kinase (Fig. 4.5) or reacylated to ceramide and recycled (134, 135). Sphingosine kinase is both a cytosolic and a membrane-associated enzyme and its activity is stimulated by treatment of cells with platelet-derived growth factor (136). Sphingosine-phosphate is acted upon by a pyridoxal 5'phosphate-dependent lyase which cleaves sphingosine-phosphate to ethanolamine phosphate and *trans*-2-hexadecenal (Fig. 4.5) (137). Ethanolamine phosphate is utilized in the synthesis of phosphatidylethanolamine and *trans*-2-hexadecenal is either oxidized to a fatty acid or reduced to the alcohol and incorporated into alkyl ether lipids.

4.4 SPHINGOLIPIDS AS MODULATORS OF CELL FUNCTION

4.4.1 Glycosphingolipids and gangliosides as modulators of cell function

In the last decade, it has become increasingly evident that GSLs are more than simply structural components of the plasma membrane and that they participate in the regulation of many important cellular processes. The observation that the content and pattern of GSLs is cell-type specific and changes during development, differentiation and oncogenesis (3-5) suggests an important role for GSLs in cell growth and proliferation. Understanding the function and mechanism of action of GSLs in these processes is an important goal of sphingolipid research.

Several techniques have been used to examine the role of GSLs and gangliosides in the function of cells. (i) One approach used extensively to examine the function of GSLs is the exogenous addition of GSLs to cells, since the amphipathic nature of GSLs permits their incorporation into cellular membranes. (ii) With the discovery and development of specific inhibitors of GSL synthesis, the effect of reducing cellular GSL levels through the inhibition of endogenous GSL synthesis has been examined. (iii) More recently, GSL expression mutants have been used as a tool to explore the function of GSLs.

GSLs and cell-cell and cell-substratum interactions

Apart from the role of GSLs as binding sites for bacterial toxins (138) and sites for microbial infection (139), a growing body of evidence suggests that GSLs, via their carbohydrate moieties, may also play a role in cell recognition and adhesion. The first hint of such a role for GSLs originates from the observation that the content and pattern of GSLs changes during embryonic development (140, 141) and that GSLs act as developmental stage-specific antigens (140, 141). Three areas of research suggest possible mechanisms of action for GSLs in cell recognition and adhesion: [i] Proteins on the surface of cells are being identified which bind to specific gangliosides. Candidate proteins include galectins (142), selectins (143) and other as yet unidentified proteins which bind to G_{M3} (144) and G_{T1b} (145). [ii] GSLs may interact with and modulate integrin receptors (146). [iii] Binding and interaction between

cells might be mediated by GSL clusters interacting with counterpart GSL clusters on the surface of another cell (146, 147).

GSLs and cellular differentiation

Since the content and pattern of GSLs change during ontogenesis and oncogenesis, investigators have speculated that GSLs and gangliosides may be involved in the process of differentiation. In support of this hypothesis, treatment of human myelocytic leukemic HL-60 cells with G_{M3} induces differentiation of these cells along a monocyte/macrophage pathway (148) whereas treatment with gangliosides of the neolacto family induces differentiation along a granulocytic pathway (149).

A role for gangliosides in neuronal differentiation is provided by evidence that exogenously administered gangliosides induce neuritogenesis and synaptogenesis in cultured neural cells (150, 151). Enhanced regeneration *in vivo* after peripheral nerve injury by treatment with exogenous gangliosides has also been observed (reviewed in 152). Conversely, antibodies raised against gangliosides inhibit neurite outgrowth from neural cells and tissues slices *in vitro* (153, 154). Recent studies by the Futerman (155, 156) and Hirabayashi (157) laboratories using inhibitors of GSL synthesis have suggested that sphingolipids play a key role in the regulation of axonal growth in hippocampal neurons (156) and dendrite growth in Purkinje neurons (157).

GSL modulation of signal transduction

Many signaling cascades are modulated by GSLs and gangliosides. For example, gangliosides have been shown to modulate calcium channel activity (158) and inhibit protein kinase C (159). More recent studies have demonstrated an interaction with and modulation of the activity of transmembrane growth factor receptors by GSLs. Both G_{M3} and G_{M1} have been shown to inhibit the proliferation of cultured cell lines, such as Swiss 3T3 cells (160, 161). The growth of most cells is dependent on the action of growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). These growth factors bind to transmembrane receptor tyrosine kinases. Both G_{M3} and G_{M1} inhibit PDGF-receptor-associated tyrosine kinase activity (160) and G_{M3} has been shown to inhibit EGF-receptor-associated tyrosine kinase activity (161). This inhibition was specific to G_{M3} and G_{M1} and not caused by other gangliosides (160, 161). These results were confirmed by experiments using G_{M3}-deficient mutant cell lines (162). The precise mechanism of ganglioside inhibition of receptor tyrosine kinases has not been elucidated but might involve the prevention of dimerization of tyrosine kinase receptors by gangliosides (146, 163).

Contrary to the negative effects of G_{M1} and G_{M3} on EGF and PDGF receptor function, a synergistic effect of G_{M1} and nerve growth factor (NGF) on neuronal survival and growth has been observed (164). Furthermore, G_{M1} alone has been demonstrated to prevent neuronal death due to NGF withdrawal (165). The effects of G_{M1} on neuronal survival and growth have been shown to be mediated by a stimulation of NGF

receptor trkA tyrosine autophosphorylation and receptor-associated protein tyrosine kinase activity (166, 167).

GSLs and the transport and sorting of GPI-anchored proteins

Glycosylphosphatidylinositol- (GPI) anchored proteins are preferentially targeted to the apical membrane of polarized Madin-Darby canine kidney cells (168). Since GPI-anchored proteins lack any sorting information, it has been suggested that sorting of GPI-anchored proteins is dependent on their interaction with GSLs, which are also enriched in the apical membrane of polarized cells (168, 169). In support of this hypothesis, both GPI-anchored proteins and GSLs have been found in detergent insoluble complexes isolated from plasma membranes (170). These complexes are thought to be specialized microdomains in the plasma membrane and are often referred to as caveolae (170, 171). A decrease in the polarized sorting of GPI-anchored proteins in Madin-Darby canine kidney cells (172) has been observed by inhibition of GSL synthesis with FB_1. These effects were reversed by removal of the inhibitor or addition of short-acyl chain ceramide effectively bypassing the block in synthesis (172). Similar results were obtained in the transport of GPI-anchored proteins in yeast (173). These results strongly suggest a role for GSLs in the transport and sorting of GPI-anchored proteins.

GSLs and gangliosides as sources of bioactive sphingolipids

Recent studies have demonstrated the bioactive properties of sphingolipid metabolites such as ceramide, sphingosine and sphingosine-phosphate and the involvement of these compounds in signal transduction events (see Section 4.4.2 below). Ceramide and sphingosine are produced by the turnover of GSLs (see Section 4.3 above) and therefore GSLs may act as a reservoir of bioactive lipids (174). It cannot be ruled out that GSLs participate in signaling events by the production of these compounds.

4.4.2 Sphingolipid metabolites as modulators of cell function

The discovery that sphingosine inhibits protein kinase C (175) was the first indication that sphingolipid metabolites might act as lipid second messengers (174). Since this discovery, a wealth of information has established that sphingolipid products participate in signal transduction and the regulation of cell function.

4.4.2.1 Ceramide

Hannun's laboratory first proposed the existence of a SM cycle (Fig. 4.6) as a new signal transduction pathway (174). In a key set of experiments they observed that SM was hydrolyzed to ceramide and phosphocholine upon treatment of HL-60 cells with 1α,25-dihydroxyvitamin D_3, an inducer of monocytic differentiation (176). These changes were transient and SM and ceramide levels returned to basal levels (176). Subsequent studies demonstrated that cell-permeant short acyl chain ceramides induced monocytic differentiation of HL-60 cells independently of 1α,25-

dihydroxyvitamin D_3 (177). Thus, ceramide was shown to be a downstream mediator of the effects of 1α,25-dihydroxyvitamin D_3 on HL-60 cells.

A number of extracellular agents which activate SM hydrolysis have been identified. Among these agents are tumor necrosis factor-α (TNF-α), endotoxin, interferon-γ, interleukin-1, Fas ligand, CD28, dexamethasone, retinoic acid, progesterone, ionizing radiation, heat and the neurotrophin family of growth factors (7, 8, 178). The time course and magnitude of ceramide accumulation in response to these agents ranges from seconds to hours (178). Acute transient production of ceramide within seconds to 1 or 2 min after treatment has been described for TNF-α, interleukin-1, ionizing radiation and Fas receptor (179-182). Small increases in ceramide levels of 20 to 50% have generally been reported in these studies (179-182). Intermediate and reversible kinetics of ceramide accumulation have been observed for 1α,25-dihydroxyvitamin D_3, the neurotrophins and also for TNF-α and interleukin-1 (176, 183-185). In these studies, ceramide levels increased to 50 to 100% above basal levels within 5 to 120 min after treatment. Finally, prolonged increases in ceramide over 12 to 48 h have been observed by TNF-α, Fas (178, 186) and serum withdrawal (187) where ceramide levels increase from 3- to 15-fold above basal levels. The explanation for the discrepancies in the time course of ceramide production reported in different studies using the same inducer is not known. Treatment of cells with cell-permeant analogs of ceramide results in large persistent increases in cellular ceramide levels which are

similar to the increases observed by serum withdrawal (187) and in the prolonged response to TNF- α and Fas (178, 186).

Ceramide is produced from SM via the action of a SMase (see Section 4.3 above). At least three SMases, differing in their pH optima, cation requirement and cellular location, have been implicated in the production of ceramide as a signaling event. 1α,25-dihydroxyvitamin D_3 has been shown to activate a cytosolic neutral Mg^{2+}-independent SMase (126). Both TNF-α (187, 188) and Fas (189) activate a neutral Mg^{2+}-dependent membrane-associated neutral SMase and an acidic SMase upon binding to their respective receptors. Activation of the acidic SMase is believed to require the production of diacylglycerol via the activation of a PC-specific phospholipase C, since an inhibitor of this phospholipase, D609, prevents the production of ceramide by TNF-α or Fas (189, 190). Domains within the cytosolic portion of the 55 kDa TNF receptor have been identified which are required for the activation of acidic or neutral SMase (188, 191, 192) and proteins which bind to these domains have been identified (193, 194).

Evidence is accumulating that distinct pools of SM are used in the production of ceramide as a cell signaling event. SM is generally believed to be localized predominantly to the outer leaflet of the plasma membrane (18, 195). However, several studies have suggested that a pool of SM on the inner leaflet of the plasma membrane is hydrolyzed by induction with 1α,25-dihydroxyvitamin D_3, TNF-α or

NGF (196-198). There is also evidence that SM within caveolae might be the pool of SM used in the agonist stimulated production of ceramide (199, 200). Caveolae are specialized domains of the plasma membrane isolated as detergent-insoluble complexes which are enriched in sphingolipids, cholesterol, GPI-anchored proteins and caveolin, a marker protein for caveolae (170, 171). Interleukin-1 stimulates the hydrolysis of SM from caveolin-rich fractions (200) and the p75 neurotrophin receptor, which mediates SM hydrolysis induced by the neurotrophins, is enriched in caveolin-rich fractions (199). Further evidence for a distinct compartment of ceramide involved in signaling is provided by studies which have shown that different techniques used to mimic the agonist induced production of ceramide, such as addition of cell-permeant ceramide, treatment with exogenous bacterial SMase and treatment with inhibitors of ceramide metabolism such as PDMP or D-myristoylamino-phenyl-propanol, do not always produce the same result (201).

Three direct molecular targets for ceramide have been identified which are believed to mediate the earliest effects of ceramide in cells (Table 4.1): ceramide-activated protein phosphatase (CAPP), ceramide-activated protein kinase (CAPK) and protein kinase C ζ. CAPP is a putative member of the protein phosphatase PP2A family (202). It is activated by ceramide and inhibited by okadaic acid *in vitro* (202, 203). Dihydroceramide, a structural analog of ceramide which lacks the effects of ceramide on cells, does not activate CAPP *in vitro* (202, 203). Some of the effects of ceramide on cells are inhibited by low concentrations of okadaic acid (204) and yeast deficient

in CAPP activity are resistant to ceramide (205). CAPK is a Mg^{2+}-dependent, plasma membrane-associated, proline-directed serine/threonine protein kinase (206). The kinase activity of CAPK is stimulated by ceramide production *in vivo* (206, 207) and recent studies suggest that one of the targets of CAPK is Raf-1 (208). Studies showing that ceramide induces phosphorylation of protein kinase C ζ in cells (209) and simulates the activity of protein kinase C ζ *in vitro* (210) suggest that this isoform of protein kinase C might be a third cellular target of ceramide.

Ceramide, suppression of proliferation and apoptosis

Cells respond to injury and stress by either undergoing cell cycle arrest or by initiating programmed cell death, depending on the severity of the insult. A role for ceramide in the suppression of cell proliferation and the induction of apoptosis is suggested by numerous studies. Most of the inducers of SM hydrolysis suppress cellular proliferation and/or induce apoptosis. The increase in ceramide induced by these agents occurs before their effects on cell growth or survival are observed (7, 8, 178). Cell permeant short acyl chain ceramides mimic the anti-proliferative and apoptotic effects of agents that activate SM hydrolysis and the magnitude of ceramide accumulation achieved by treatment of cells with short acyl chain ceramides is comparable to the levels attained by the prolonged response to TNFα, Fas or serum deprivation (178, 186, 187). Dihydroceramide, which differs from ceramide only in the lack of a 4,5 *trans* double bond, does not have these effects on cells, demonstrating the specificity of the ceramide response (211). Alternate methods used

to increase intracellular ceramide levels including treatment of cells with exogenous bacterial SMase or inhibitors of ceramide metabolism, can also result in the inhibition of cellular proliferation and/or the induction of apoptosis (7, 8, 90, 133).

Ceramide modulates signaling pathways which are known to be involved in cell proliferation and apoptosis (Table 4.1). Dephosphorylation of the retinoblastoma protein (212), suppression of the expression of the c-myc protooncogene (213), inactivation of protein kinase Cα (214) and inhibition of the cellular activation of phospholipase D (215) are documented responses to elevation of ceramide levels which might contribute to the anti-proliferative effect of ceramide on cells. In addition, some investigations have suggested that ceramide induces nuclear translocation and activation of NF-κB (190, 216), a transcription factor involved in the control of apoptosis and stress responses (217). This is a much debated issue, however, since several studies have failed to observe an effect of ceramide on NF-κB (218, 219). Ceramide activates proteases of the ICE-like family which are known to be activated during the induction of apoptosis (220, 221). Bcl-2, an anti-apoptotic protein, inhibits the ability of ceramide to activate these proteases and prevents ceramide-mediated apoptosis (220, 222). A major signaling pathway which is activated in response to cellular stress is the stress-activated protein kinase (SAPK) pathway (223). Ceramide has been shown to activate the SAPK cascade (224, 225) and, therefore, a link might exist between ceramide production and the SAPK stress-response pathway.

Ceramide and differentiation

The first cellular response to ceramide reported was its ability to induce the differentiation of HL-60 cells along a monocytic pathway (176). A number of additional cell types, including gliomas (184) and neuroblastomas (226), have been shown to undergo differentiation in response to elevations in intracellular ceramide.

Ceramide and the nervous system

The discovery that nerve growth factor and other neurotrophins can activate SM hydrolysis via the p75 neurotrophin receptor ($p75^{NTR}$) (184, 185) suggests that this might be an important signaling pathway in nervous tissue (227). The $p75^{NTR}$ belongs to a family of receptors which also includes the TNF and Fas receptors (228, 229). This receptor is widely distributed in cells of the brain and binds all members of the neurotrophin family, which includes NGF, brain-derived neurotrophic factor and neurotrophin-3/4/5, with similar affinities (228, 229). The neurotrophins also bind to members of the trk receptor tyrosine kinase family and association with trk receptors has been shown to be required in the functioning of neurotrophins as neurite growth and cell survival promoting factors (reviewed in 230 and 231). The role of $p75^{NTR}$ in neurotrophin function, however, has not been clearly elucidated and the SM cycle is the first signaling pathway found to be coupled to this receptor (184). Several recent studies have reported a role for the neurotrophin receptor in the induction of apoptosis (232-235). The role of ceramide in this process remains to be evaluated. Certain

populations of neurons are susceptible to the apoptotic effects of ceramide (236, 237). In other neurons ceramide seems to have a protective effect (238, 239). Additional studies have demonstrated that ceramide synthesis is required for normal axonal growth of hippocampal neurons (156) and dendrite growth of Purkinje neurons (157). An important goal of sphingolipid research is, therefore, to determine the function of ceramide in the nervous system and in particular its role in the function of $p75^{NTR}$.

4.4.2.2 Sphingosine and sphingosine-phosphate

The discovery that sphingosine inhibits protein kinase Cα *in vitro* and in intact cells (175) was the first indication that sphingosine might act as an intracellular effector. In many cells, sphingosine has an anti-proliferative effect which stems from the inhibition of protein kinase C-mediated processes (33, 240). In contrast to the growth suppressive effects of sphingosine on most cells, a mitogenic effect was observed by treatment of growth arrested Swiss 3T3 fibroblasts with sphingosine (241). Sphingosine taken up by these fibroblasts was rapidly converted to sphingosine-phosphate by sphingosine kinase (242). Platelet-derived growth factor, which stimulates the cellular proliferation of Swiss 3T3 cells, induces rapid and transient increases in both sphingosine and sphingosine-phosphate, suggesting that these lipids may be involved in the mitogenic effect of platelet-derived growth factor (136). Many cellular responses to elevations in sphingosine and sphingosine-phosphate have been reported (Table 4.1) including stimulation of phospholipase D (243), inhibition of phosphatidate phosphohydrolase (244), mobilization of Ca^{2+} from internal stores

(240) and reduction of cyclic AMP levels (241). All of these effects of sphingosine and sphingosine-phosphate may contribute to their mitogenic effect of Swiss 3T3 fibroblasts.

Evidence is accumulating that suggests that the balance of sphingolipid metabolites, ceramide versus sphingosine and sphingosine-phosphate, affects the growth and survival of a cell (Table 4.1). Ceramide can suppress the mitogenic effects of sphingosine and sphingosine-phosphate and *vice versa* (245). The induction of apoptosis by ceramide can be blocked by sphingosine-phosphate (246). Ceramide and sphingosine have been shown to differentially affect the mitogen-activated protein kinase and stress-activated protein kinase cascades (247). Ceramide activates SAPK (224, 225, 247), whereas sphingosine activates the mitogen activated protein kinase cascade (247). Furthermore, growth factors have been shown to induce alkaline ceramidase activity (248) which would be expected to increase the sphingosine content and decrease the ceramide content of a cell. It is plausible that a cell might regulate the balance between mitogenic (sphingosine and sphingosine-phosphate) and anti-mitogenic (ceramide) sphingolipid metabolites according to its growth characteristics.

4.5 Thesis Objective: Part II

One of the goals of our laboratory is to examine the synthesis, transport and function of lipids in neurons, in particular in the process of axonal growth and regeneration.

Studies in the second half of this thesis were, therefore, initiated in order to examine the synthesis and role of GSLs in axonal growth. As mentioned in Section 4.4.1 above, many investigations have suggested that gangliosides play an important role in neurite growth since the exogenous addition of gangliosides to neural cells induces neuritogenesis and synaptogenesis (150, 151). Inhibition of neurite growth was observed when antibodies raised against gangliosides were supplied to neural cells and tissues slices *in vitro* (153, 154). In Chapter 5, the effect of reducing, instead of augmenting, endogenous gangliosides on axonal growth was determined using inhibitors of GSL synthesis. Results from studies in Chapter 5 suggest that newly synthesized GSLs are not required for axonal growth but that the lipid second messenger ceramide might negatively regulate axonal growth. Elevation of ceramide within the distal axons of rat sympathetic neurons inhibited axonal growth. The goal of studies in Chapter 6 was to begin to characterize and elucidate the mechanism(s) of ceramide-mediated inhibition of axonal growth.

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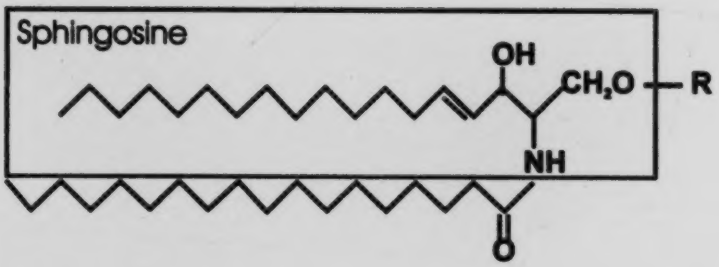

Fig. 4.1 **Structure of sphingolipids**

A sphingolipid consists of a sphingoid base, an amide linked fatty acid and a headgroup (R). The most common sphingoid base in mammalian sphingolipids is sphingosine. The headgroup (R) = H for sphingosine or ceramide, phosphocholine for SM or a carbohydrate chain for the GSLs.

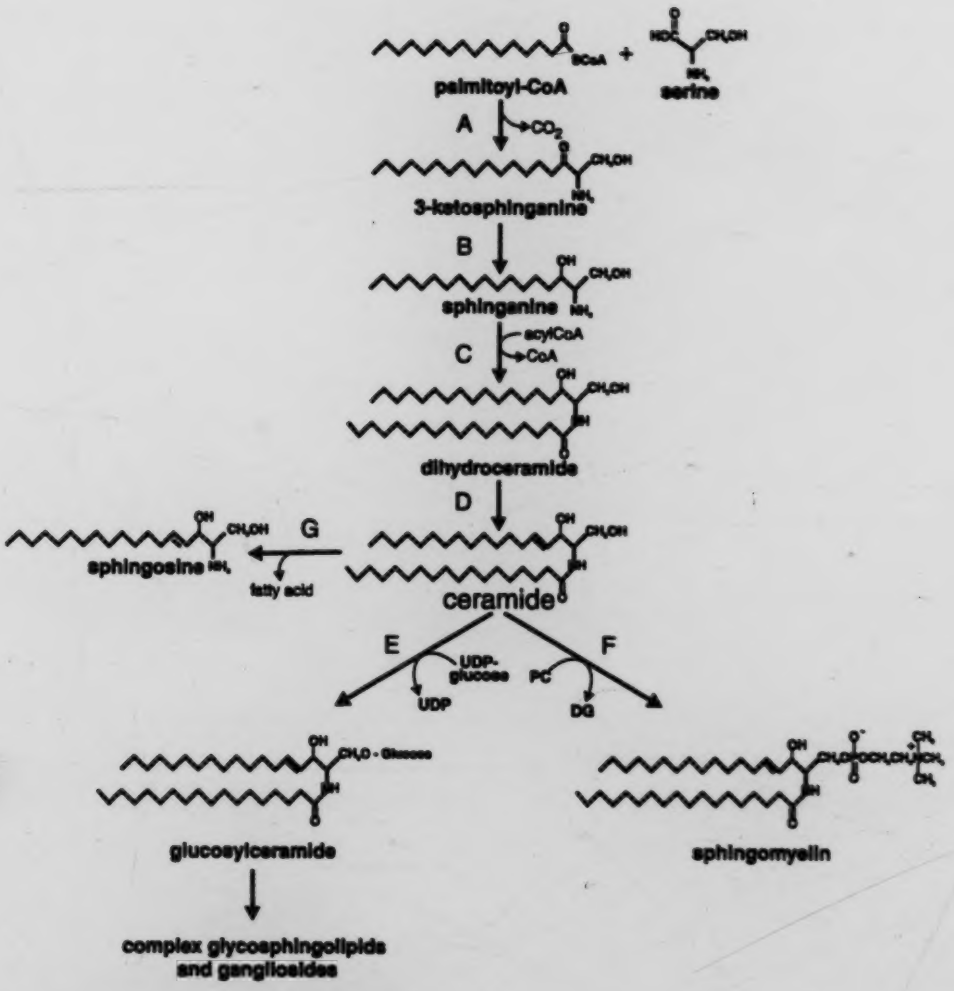

Fig. 4.2 **Biosynthesis of sphingolipids**

A, serine palmitoyltransferase; B, 3-ketosphinganine reductase; C, sphinganine *N*-acyltransferase; D, dihydroceramide desaturase; E, glucosylceramide synthase; F, PC:ceramide cholinephosphotransferase; G, ceramidase.

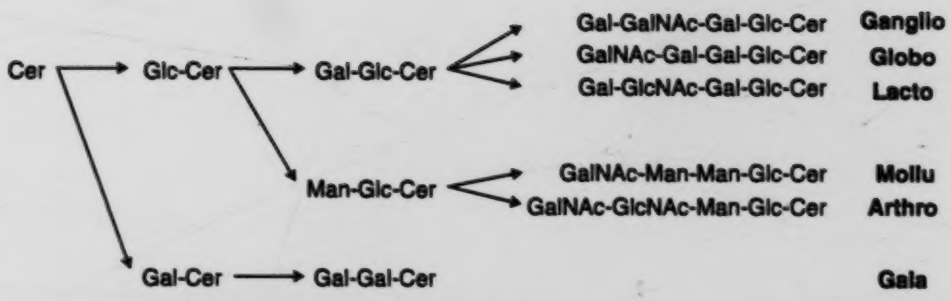

Fig. 4.3 **Biosynthesis of the oligosaccharide core structure of glycosphingolipids**

Glycosphingolipids can be classified into series or families (Ganglio, Globo, Lacto, Mollu, Arthro, Gala) based on the core structure of their oligosaccharide chain. Cer, ceramide; Glc, glucose; Gal, galactose; Man, mannose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine.

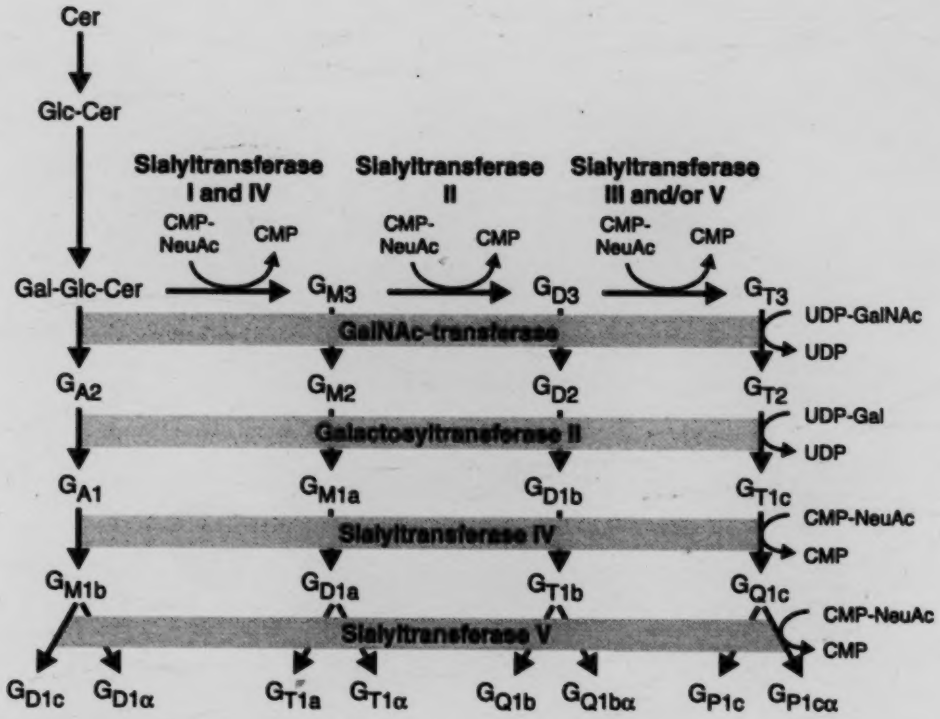

Fig. 4.4 **Biosynthesis of gangliosides of the ganglio family**

Gangliosides are synthesized by the stepwise glycosylation or sialylation of the growing carbohydrate chain attached to ceramide. A relatively small number of glycosyltransferases are needed to synthesize the wide range of gangliosides of this family. Cer, ceramide; Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; CMP-, cytidine monophosphate-; UDP-, uridine diphosphate-. Adapted from (194).

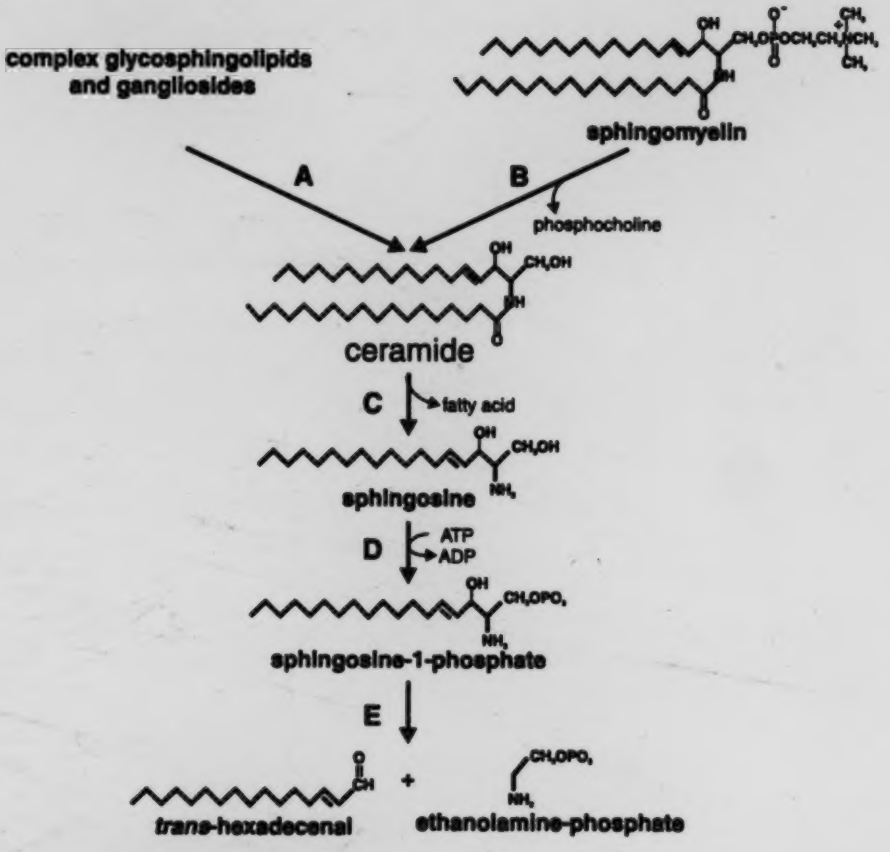

Fig. 4.5 **Degradation of and sphingolipids**

A, glycosidases; B, sphingomyelinase; C, ceramidase; D, sphingosine kinase; E, sphingosine-1-phosphate lyase.

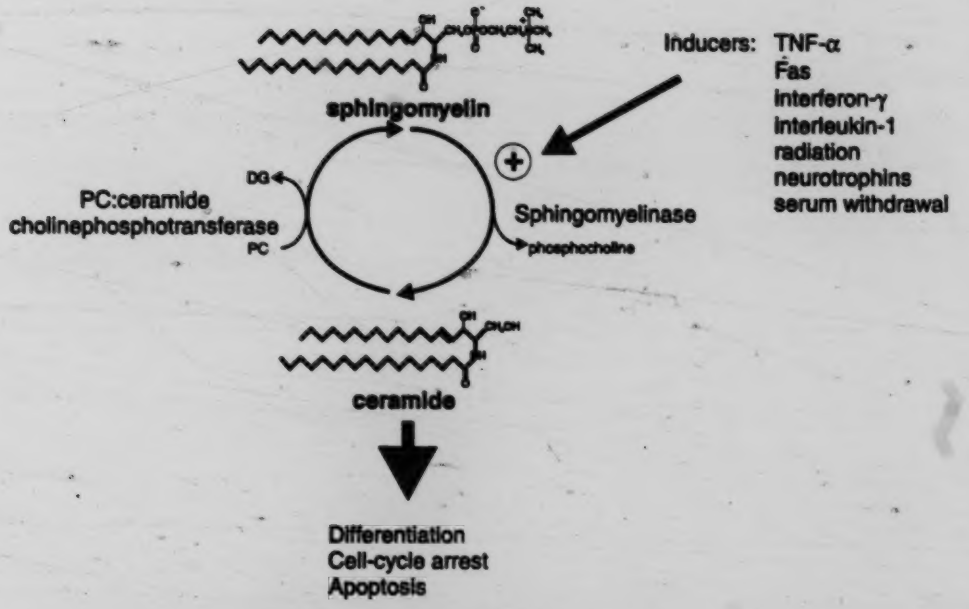

Fig. 4.6 Sphingomyelin signal transduction pathway

A schematic illustration of signaling through ceramide. The interaction of a number of extracellular agents with their receptors, as well as certain environmental conditions, induces the activation of sphingomyelinases which hydrolyze plasma membrane sphingomyelin releasing ceramide. Ceramide activates a signaling cascade which can ultimately result in the induction of differentiation, cell-cycle arrest or apoptosis.

Table 4.1 **Targets, cellular effects and biological responses to ceramide, sphingosine and sphingosine-phosphate**

CAPP, ceramide-activated protein phosphatase; CAPK, ceramide-activated protein kinase; PKC, protein kinase C; Rb, retinoblastoma protein; PLD, phospholipase D, SAPK, stress-activated protein kinase; ICE, interleukin-1 converting enzyme; PAP, phosphatidate phosphohydrolase; cAMP, cyclic adenosine monophosphoate; MAPK, mitogen activated protein kinase.

	Direct targets	Cellular Effects	Biological Response
ceramide	CAPP	dephopshorylation of Rb	differentiation
	CAPK	suppression of c-myc	cell cycle arrest
	PKCζ	inactivation of PKCα	apoptosis
		inhibition of PLD	
		activation of SAPK	
		activation of ICE proteases	
sphingosine &		stimulation of PLD	cell proliferation
sphingosine-1-P		inhibition of PAP	survival
		mobilization of Ca^{2+}	
		reduction of cAMP levels	
		activation of MAPK	

CHAPTER 5

ELEVATION OF CERAMIDE WITHIN DISTAL AXONS INHIBITS AXONAL GROWTH OF RAT SYMPATHETIC NEURONS

The majority of this work was published in
J. Biol. Chem. (1997) **272**, 3028-3035.

5.1 INTRODUCTION

GSLs are major components of eukaryotic cell membranes and are particularly enriched in neuronal membranes (See Chapter 4 Section 4.1). They are located predominantly in the outer leaflet of the plasma membrane (1) where they have been postulated to play a role in a number of important cellular processes including cell-cell and cell-substratum recognition, adhesion, differentiation, proliferation and oncogenic transformation. The pattern of GSLs differs among cell types and changes during development, cellular differentiation and oncogenic transformation suggesting an important role for GSLs in cell growth and proliferation (2-4). In addition, intermediates in the biosynthesis and catabolism of sphingolipids and GSLs may function as lipid second messengers mediating the effects of extracellular agents and agonists (5-7).

One approach used extensively to examine the function of GSLs is the exogenous addition of GSLs to cells, since the amphipathic nature of GSLs permits their incorporation into cellular membranes. The enrichment of gangliosides (sialic-acid containing GSLs) in neuronal membranes induces neuritogenesis (2, 8-11), modulates growth factor receptor activity (2, 12, 13), potentiates responses to neurotrophic factors (12, 14) and protects against apoptotic death caused by withdrawal of trophic support (15). Moreover, antibodies raised against gangliosides inhibit neurite outgrowth from neural cells and tissues slices *in vitro* (16, 17). Tettamanti and Riboni

(18) have summarized the role of gangliosides in neurodifferentiation, neuritogenesis and synaptogenesis.

Only recently has the effect of the reduction of cellular GSL levels on neurons through the inhibition of endogenous GSL synthesis been examined. Inhibition of dihydroceramide synthesis, an early step in the synthesis of all GSLs (see Chapter 4, Fig. 4.2), in cultured hippocampal neurons disrupts axonal growth (19) and the formation and maintenance of axonal branches (20). Similar studies have found that sphingolipid biosynthesis is essential for dendrite growth and survival of cerebellar Purkinje cells (21).

In the present study, the role of GSLs in axonal growth was investigated using two inhibitors of endogenous GSL synthesis. The first inhibitor, fumonisin B_1 (FB_1) (Fig. 5.1), has been demonstrated to inhibit sphinganine *N*-acyltransferase (see Chapter 4 Section 4.2.1 and Fig. 4.2) in a variety of cell types such as rat hepatocytes (22), LLC-PK_1 pig kidney cells (23) and hippocampal neurons (19). The second inhibitor, PPMP (D,L-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) (Fig. 5.1), is a potent inhibitor of glucosylceramide synthesis (see Chapter 4 Section 4.2.3 and Fig. 4.2). A less active analogue of PPMP, PDMP (D,L-1-phenyl-2-decanoylamino-3-morpholino-propanol), blocks GSL synthesis in cultured 3T3 cells (24), Madin-Darby canine kidney cells (25) and hippocampal neurons (20). Although PPMP and PDMP inhibit

the synthesis of glucosylceramide in cellular homogenates to a similar extent, PPMP has 10 times the potency of PDMP in intact cells (25).

Evidence is presented in this Chapter that both FB_1 and PPMP inhibit GSL synthesis in cultured primary rat sympathetic neurons. The results suggest, however, that newly synthesized GSLs are not essential for axonal growth but that the lipid second messenger ceramide negatively regulates axonal growth.

5.2 Materials and Methods

[9,10-^{3}H]Palmitate (specific activity 54 Ci/mmol) was purchased from Amersham Canada, Oakville, ON, Canada. FB_1 was generously provided by Dr. A. Merrill, Emory University, Atlanta or purchased from Sigma Chemical Co. (St. Louis, MO). *threo*-PPMP, *erythro*-PPMP, C_6-ceramide and C_6-dihydroceramide were purchased from Matreya Inc., Pleasant Gap, PA. NBD-C_6-ceramide was purchased from Molecular Probes, Inc. Eugene, OR (USA). Standard phospholipids were isolated from rat liver or purchased from Avanti Polar Lipids, Birmingham, AL. Ganglioside standards were kindly supplied by Dr. R. Yu, Medical College of Virginia, Richmond, VA. All other reagents and chemicals used are listed in Chapter 2 Section 2.2 or Chapter 3 Section 3.2.

Preparation of Neuronal Cultures

Mass cultures of rat sympathetic neurons were prepared as described in Chapter 2 Section 2.2 and compartment cultures were prepared as described in Chapter 3 Section 3.2.

Treatment of Neuronal Cultures

FB_1, *erythro*-PPMP and *threo*-PPMP were dissolved in water to make 10 mM stock solutions. C_6-Ceramide and C_6-dihydroceramide were dissolved in dimethylsulfoxide to make 10 mM stock solutions. These stock solutions were used to prepare medium containing the desired final concentration of inhibitor or ceramide. The final concentration of dimethylsulfoxide in the culture medium of cells treated with C_6-ceramide or C_6-dihydroceramide never exceeded 0.25%. Control cultures were given a similar aliquot of dimethylsulfoxide.

Measurement of *de novo* GSL Biosynthesis

The effect of FB_1 and PPMP on GSL synthesis was determined by preincubating neurons cultured in 24-well dishes in the presence or absence of the indicated concentration of inhibitor for 4 d. [^{3}H]Palmitate (10 μCi/ml) was present during the last 24 h of incubation. The radiolabeled medium was aspirated and the neurons washed twice with ice-cold phosphate-buffered saline (pH 7.4). Cellular material was collected in methanol/water (8:3, v/v) and sonicated for 10 s using a probe sonicator. Chloroform was added to give a final chloroform/methanol/water ratio of 4:8:3 (v/v).

The solvent mixture was evaporated to dryness and the gangliosides and phospholipids were extracted with diisopropyl ether/butanol/50 mM NaCl (6:4:5, v/v) as described by Ladisch and Gillard (26). Under these conditions gangliosides are recovered in the lower aqueous phase. The samples were desalted by passage through a Sephadex G-50 column. Gangliosides were separated, along with authentic standards, by high performance thin-layer chromatography in the solvent system chloroform/methanol/15 mM $CaCl_2$ (55:45:10, v/v). The plates were sprayed with resorcinol-HCl-Cu^{2+} reagent (27) and heated in a 180°C oven for 20 min. The bands corresponding to authentic standards of G_{M1}, G_{M3}, and G_{T1b} were scraped and the radioactivity incorporated measured by liquid scintillation counting. The phospholipids PC and SM were separated by thin-layer chromatography in the solvent system chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:1, v/v). Bands corresponding to authentic PC and SM were scraped and the radioactivity incorporated measured by liquid scintillation counting. Ceramide was separated by thin-layer chromatography in two consecutive solvent systems: chloroform/methanol/acetic acid (9:1:1, v/v) followed by petroleum ether/diethyl ether/acetic acid (60:40:1, v/v). The band corresponding to authentic ceramide was scraped and the radioactivity incorporated measured by liquid scintillation counting. In all cases, radioactive incorporation was normalized to total phospholipid mass.

Measurement of Axonal Extension

Axonal extension was measured in compartmented cultures. Axotomy was performed by mechanical removal of the distal axons from left and right compartments with a jet of sterile distilled water delivered with a syringe through a 22-gauge needle. The water was aspirated and the wash repeated twice followed by the addition of fresh culture medium. This procedure effectively removes all visible traces of axons from the side compartments (28). Measurements of axonal growth were made as previously described (28) using a Nikon Diaphot inverted microscope with phase contrast optics outfitted with a MD2 microscope digitizer (Minnesota Datametrics Corp., Minneapolis, MN) which tracks stage movements to an accuracy of ± 5 μm. An on-line computer using custom software (Minnesota Datametrics Corp.) calculated the distance from the edge of the silicone grease to the farthest extending axon on each track and combined these measurements to obtain means and standard errors. In each culture, axons in 16 tracks were measured in left and right compartments.

Metabolism of Fluorescent Ceramide

13 day-old neurons cultured in compartmented dishes were given 10 μM NBD-C_6-ceramide in the distal axon-containing compartment. After 24 h the medium was aspirated and the axons were washed three times with ice-cold phosphate-buffered saline (pH 7.4). The cellular material was collected, the lipids were extracted (29) and separated by thin-layer chromatography using a 3-solvent system (30). Spots corresponding to authentic standards of ceramide, ceramide-1-phosphate and SM

were scraped, extracted from the silica and quantified using a Hitachi F-2000 fluorimeter (excitation wavelength = 464 nm, emission wavelength = 532 nm).

Other Methods

The phospholipid content of cells was measured by lipid phosphorous determination (31).

5.3 Results

5.3.1 Inhibition of *de novo* sphingolipid biosynthesis in sympathetic neurons

The effect of FB_1 and PPMP on sphingolipid synthesis in rat sympathetic neurons was examined. 11-day-old neurons, cultured in 24-well dishes, were incubated with various concentrations of FB_1, *threo*-PPMP or *erythro*-PPMP for 4 days. [^{3}H]palmitate (10 μCi/ml) was added for the last day of incubation and the incorporation of radiolabel into gangliosides was measured. There was no advantage to using compartmented cultures for this study since when [^{3}H]palmitate is supplied to distal axons the label rapidly equilibrates throughout the neurons. Fig. 5.2 shows that both FB_1 and *threo*-PPMP inhibited the incorporation of [^{3}H]palmitate into G_{M1} in a dose-dependent manner. As expected, *erythro*-PPMP did not reduce the incorporation of [^{3}H]palmitate into G_{M1} since the *erythro* isomer does not inhibit sphinganine *N*-acyltransferase (32). Table 5.1 summarizes the effect of FB_1 (25 μM) and PPMP (5 μM) on the incorporation of [^{3}H]palmitate into several gangliosides, namely G_{M1}, G_{M3} and G_{T1b}. The incorporation of [^{3}H]palmitate into all three

gangliosides was greatly reduced by treatment with 25 μM FB_1 or 5 μM *threo*-PPMP, but not *erythro*-PPMP, indicating that FB_1 and *threo*-PPMP are effective inhibitors of GSL synthesis in rat sympathetic neurons.

5.3.2 Glycosphingolipids and axonal growth

Since FB_1 and PPMP inhibited GSL synthesis in rat sympathetic neurons, the response of axonal growth to these inhibitors was investigated. Neurons were plated in the center compartment of compartmented culture dishes and allowed to grow for 11 days. At that time, medium containing 5 μM PPMP was given to either the center (cell body-containing) compartment alone, or to the side (distal axon-containing) compartments. Control cultures were given medium without PPMP. After 3 days distal axons were removed from the left and right compartments (axotomy) and the cells were incubated as before. Axonal extension was measured every day for the following 4 days (Fig. 5.3). In cultures that had been given *threo*-PPMP in the distal axon-containing compartments alone, 3.7 days after axotomy axonal extension was 61% less than that of cultures given medium lacking PPMP (Fig. 5.3A). In contrast, the growth of axons of cultures given *threo*-PPMP to the cell body-containing compartment alone was unaffected and the axons elongated at the same rate as did untreated cells. The effect of *threo*-PPMP on axonal growth was most likely specifically related to the inhibition of glucosylceramide synthase since the *erythro* isomer of PPMP did not affect axonal elongation regardless of whether it was added to the cell body- or to the distal axon-containing compartments (Fig. 5.3B). Only in

those cultures to which *threo*-PPMP had been added to the distal axons was axonal growth impaired. In a separate experiment, the same effect (*i.e.* inhibition of distal axon growth) was observed when cultures were treated with *threo*-PPMP in both center and side compartments at the same time. The axons of untreated cultures elongated at a rate of 1.00 ± 0.08 mm/day whereas the rate of axonal elongation in cultures treated with 5 μM *threo*-PPMP was 0.21 ± 0.05 mm/day when *threo*-PPMP was supplied to the side compartments alone and 0.32 ± 0.03 mm/day when *threo*-PPMP was supplied to all compartments.

To exclude a general toxic effect of *threo*-PPMP on sympathetic neurons, some cultures that had been incubated with *threo*-PPMP in the distal axon-containing compartments for the first 3 days were given medium lacking the inhibitor after axotomy. Axonal growth resumed at approximately the normal rate (0.7 mm/day for PPMP-treated cultures vs. 0.8 mm/day for untreated neurons) indicating that the effect of *threo*-PPMP on axonal growth was reversible. Moreover, *threo*-PPMP did not exert a direct cytotoxic effect when given to the cell body-containing compartment alone, since axons in these cultures extended at the same rate as axons in untreated cultures (Fig. 5.3A).

The effect of FB_1 on axonal elongation was also investigated. Sympathetic neurons, cultured for 11 days in compartmented dishes, were given 25 μM FB_1 either in the center, cell body-containing compartment alone or in the distal axon-containing

compartments alone. After 3 days, cultures were axotomized and given FB_1 in either the side or center compartments, as before. Control cultures were given medium without FB_1 in all compartments. Axonal extension was subsequently measured every 24 h. Axonal growth in cultures given FB_1 to the cell body-containing compartment alone, to the distal axon-containing compartments alone (Fig. 5.4) or to both compartments (rate of axonal elongation 0.98 ± 0.05 mm/day) was identical to that in control cultures. This result was unexpected since in these neurons 25 μM FB_1 inhibited GSL synthesis to a similar extent to that of *threo*-PPMP (Fig. 5.2 and Table 5.1). When cells were treated with a higher concentration of FB_1 (50 μM) in the cell body- or the distal axon-containing compartments, axonal extension was 28 and 21% less than in control cells, respectively. However, this effect was probably due to cytotoxicity since cells incubated for 3 days in the presence of 50 μM FB_1, and transferred after axotomy to medium containing no FB_1, did not recover normal axonal growth and died within 3 days.

Culture medium supplemented with 2.5% rat serum, which may be a source of some sphingolipids (33), is routinely given to the cell body-containing compartment. Thus, possible explanations for the observed lack of effect of the inhibitors when supplied to the cell body-containing compartment were either that sphingolipids were present in serum or that the inhibitors bound albumin, reducing the effective concentration of FB_1 or PPMP in the medium. Medium containing 1% delipidated rat serum, or medium completely lacking serum, was used to minimize the possible interference of

exogenously added lipids. Results obtained under these conditions were identical to those obtained using medium containing 2.5% rat serum, *i.e.* axonal growth was impaired only in cultures treated with *threo*-PPMP in distal axons and FB_1 did not affect axonal elongation.

Since *threo*-PPMP impaired axonal growth, whereas FB_1 did not, it is concluded that *threo*-PPMP is acting on axonal growth via a mechanism other than inhibition of GSL synthesis. In addition, the results suggest that newly synthesized GSLs are not required for axonal growth.

5.3.3 FB_1 decreases, whereas PPMP increases, labeled ceramide

The experiments presented in Fig. 5.2 and Table 5.1 demonstrate that both FB_1 and PPMP inhibit GSL synthesis in rat sympathetic neurons. However, because FB_1 and PPMP inhibit different enzymes (see Chapter 4 Sections 4.2.1 and 4.2.3 and Fig. 4.2) it is anticipated that the two inhibitors would differentially affect ceramide levels: FB_1 would be expected to decrease the level of ceramide, whereas PPMP treatment would result in ceramide accumulation. In rabbit skin fibroblasts, *threo*-PDMP has been reported to increase the intracellular mass of ceramide (34). Alternatively, one might expect that PPMP would increase SM levels since *threo*-PDMP enhances the synthesis of labeled SM and ceramide from [^{3}H]palmitate in 3T3 cells (35) and causes SM accumulation in hippocampal neurons (20). The effect of FB_1 and PPMP on the incorporation of [^{3}H]palmitate into SM, ceramide and PC in rat sympathetic neurons

was, therefore, investigated. Neurons were treated for 4 days according to the protocol used in the experiment depicted in Fig. 5.2. [^{3}H]Palmitate was added during the last 24 h and the amount of radioactivity incorporated into SM, ceramide and PC was measured. FB_1 treatment decreased the incorporation of [^{3}H]palmitate into SM by ~50% (Fig. 5.5C). However, neither isomer of PPMP affected the incorporation of radioactivity into SM (Fig. 5.5D). Treatment of the neurons with FB_1 inhibited the incorporation of [^{3}H]palmitate into ceramide (Fig. 5.5A), which might explain the reduced incorporation of radioactivity into SM (Fig. 5.5B). On the other hand, treatment of neurons with *threo*-PPMP, but not *erythro*-PPMP, increased the incorporation of [^{3}H]palmitate into ceramide (Fig. 5.5B). The effects of FB_1 and PPMP on the incorporation of [^{3}H]palmitate into SM and ceramide were specific since the incorporation of radiolabel into PC was not affected by either FB_1 or PPMP (Fig. 5.5, E and F).

5.3.4 Exogenously-added ceramide inhibits axonal extension

Since *threo*-PPMP, but not *erythro*-PPMP or FB_1, inhibited axonal extension in rat sympathetic neurons (Figs. 5.3 and 5.4), and *threo*-PPMP elevated labeled ceramide levels within the neurons (Fig. 5.5), the possibility that ceramide might be responsible for the growth inhibitory effect of *threo*-PPMP was considered. If this were the case, pretreatment of neurons with FB_1 might be expected to prevent the accumulation of ceramide that occurred when cells were incubated with *threo*-PPMP (see Chapter 4 Sections 4.2.1 and 4.2.3 and Fig. 4.2) and might thereby eliminate the inhibitory

effect of *threo*-PPMP on axonal growth. To test this hypothesis, cultures were either left untreated for 7 days, left untreated for 4 days and then treated with 5 μM *threo*-PPMP for 3 days or treated with 25 μM FB_1 for 4 days and then treated with 5 μM *threo*-PPMP for 3 days. Cultures were axotomized and allowed to regenerate under pre-axotomy conditions. The rate of axonal extension of untreated cultures was 2.65 ± 0.04 mm (Fig. 5.6). In cultures provided with *threo*-PPMP axonal growth was inhibited (extension was 0.93 ± 0.03 mm), as occurred in the experiment depicted in Fig. 5.3. However, axonal extension of neurons given both FB_1 and *threo*-PPMP was very similar to that of neurons incubated without either inhibitor (2.48 ± 0.06 mm) (Fig. 5.6). The results of this experiment support the idea that ceramide inhibits axon growth.

As additional evidence that ceramide inhibits axonal extension, the effect of cell-permeable, short-acyl chain (6 carbons) ceramide (C_6-ceramide) on axonal extension was determined. Sympathetic neurons were plated in three-compartment dishes and allowed to grow for 14 days after which 10 μM C_6-ceramide or 10 μM *erythro*-C_6-dihydroceramide was supplied to either the cell body-containing compartment alone or the distal axon-containing compartments alone. After 2 days the cells were neuritotomized and re-incubated with C_6-ceramide or *erythro*-C_6-dihydroceramide, then axonal extension was measured every day for the following 5 days. Fig. 5.7A shows that neurons treated with C_6-ceramide in the cell body-containing compartment alone elongated at the same rate as did untreated cells. On the other hand, treatment of

neurons with C_6-ceramide in the distal axon-containing compartments alone inhibited axonal elongation by 60%. In a separate experiment, axonal growth was also inhibited when cultures were treated with C_6-ceramide simultaneously in all compartments. The axons of untreated cultures elongated at a rate of 1.15 ± 0.08 mm/day whereas axons of cultures treated with 10 μM C_6-ceramide in all compartments elongated at a rate of 0.21 ± 0.05 mm/day. These results correlate with the effect of *threo*-PPMP on axonal growth (Fig. 5.3A), indicating that ceramide might mediate the inhibitory effect of *threo*-PPMP on axonal growth.

A possible explanation for the observed lack of an effect of ceramide when supplied to the cell body-containing compartment alone was that ceramide might have bound to components of serum present in the medium and thereby reduce the effective concentration of available ceramide. To eliminate this possibility the experiment depicted in Fig. 5.7 was repeated, but with medium lacking rat serum. Axonal extension 2.5 days after axotomy was 2.04 ± 0.04 mm for cells incubated without ceramide and 2.15 ± 0.04 mm for neurons given 10 μM ceramide in the cell body-containing compartment, demonstrating that when C_6-ceramide is given to cell bodies/proximal neurites extension of distal axons is unaffected.

Bielawska *et al.* (36) have previously shown that C_2-ceramide inhibits the growth of HL-60 human myelocytic leukemia cells and induces apoptosis whereas *erythro*-C_2-dihydroceramide is inactive. The effect of *erythro*-C_6-dihydroceramide on axonal

growth was, therefore, examined. *Erythro*-C_6-dihydroceramide (10 μM) did not inhibit axonal elongation when given to either the cell body-containing compartment or the distal axon-containing compartments (Fig. 5.7B), confirming the specificity of the inhibitory effect of C_6-ceramide on axon elongation.

Dioctanoylglycerol (diC_8-glycerol) is a cell-permeant analog of diacylglycerol, a known antagonist of the effects of ceramide on viability/apoptosis (6, 37). It was therefore determined if diC_8-glycerol reversed the ceramide-mediated inhibition of axonal growth. Rat sympathetic neurons were plated in compartmented dishes for 13 days. Distal axons were removed and allowed to regenerate for 1 day, after which the right compartments were supplied with medium containing either 10 μM C_6-ceramide or 30 μM diC_8-glycerol or both compounds simultaneously. In all cultures, the left compartment contained neither C_6-ceramide nor diC_8-glycerol and acted as a control. Axonal extension was measured immediately before treatment and for 2 days following treatment. Axons in right side compartments that were given C_6-ceramide retracted slightly after 24 h treatment and did not resume normal growth (axonal elongation before treatment was 0.83 ± 0.03 mm and 1.8 days after treatment was 0.55 ± 0.03 mm). Similar results were obtained in cultures given diC_8-glycerol together with C_6-ceramide (axonal elongation before treatment was 0.90 ± 0.03 mm and 1.8 days after treatment was 0.55 ± 0.03 mm). Axons in the left (control) compartment elongated at a rate of 1.06 ± 0.07 mm/day. Axons in right side compartments treated with diC_8-glycerol alone elongated at the same rate as control

axons. It was not possible to assess whether or not higher concentrations of diC_8-glycerol reversed the inhibitory effect of ceramide on axonal growth because 100 μM diC_8-glycerol inhibited axonal elongation. These results suggest that mechanisms involved in ceramide-mediated inhibition of axonal growth differ from those involved in ceramide-mediated induction of apoptosis. The experiments also show that ceramide acts rapidly on axonal extension and suggest that the delay in the effect of PPMP is due to the time required for accumulation of ceramide within the axons.

Figure 5.8 represents photomicrographs of cultures treated with C_6-ceramide as indicated in the previous experiment. The *bottom panel* shows the right side compartment 24 h after treatment with 10 μM C_6-ceramide. The C_6-ceramide-treated axons were significantly shorter than were untreated neurons (*top panel*) and were abnormal in appearance. Cell bodies and proximal neurites in the center compartment appeared normal (*center panel*). Since cell bodies looked morphologically normal, even when directly exposed to 10 μM C_6-ceramide (not shown), it is concluded that under the conditions used C_6-ceramide did not induce apoptosis.

5.3.5 Ceramide metabolism

To determine whether the inhibition of axonal growth by PPMP and C_6-ceramide was due to ceramide itself or to a metabolite of ceramide, the metabolism of a fluorescent, short-chain analogue of ceramide, C_6-NBD-ceramide (*N*-{6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl}-D-*erythro*-sphingosine) was studied. Experiments were

first carried out to demonstrate that C_6-NBD-ceramide exhibited the same growth inhibitory properties as C_6-ceramide. 14-Day old sympathetic neurons cultured in compartmented dishes were axotomized and given 10 μM C_6-NBD-ceramide in the right side compartment. The left compartment was given medium lacking ceramide as a control. After 24 h, axons elongated by 1.03 ± 0.08 mm, whereas axons treated with C_6-NBD-ceramide extended only 0.30 ± 0.04 mm, indicating that this analogue behaved as did C_6-ceramide in terms of growth inhibition.

To analyze the metabolism of C_6-NBD-ceramide in rat sympathetic neurons, 14-day old neurons were incubated with 10 μM C_6-NBD-ceramide in the distal axon-containing compartments for 24 h, after which axons were harvested and lipids extracted and isolated. Of the total cell-associated fluorescence 95% was recovered in ceramide, 2.7% was in ceramide-1-phosphate and 0.7% was in SM, showing that in the axons of sympathetic neurons, as in other cell types (20, 38, 39), short-chain ceramide is inefficiently metabolized. It is likely that the small unrecovered percentage of fluorescence is present in the form of glucosylceramide or hexanoate derived from the hydrolysis of ceramide to sphingosine. Most likely, therefore, ceramide itself, rather than one of its metabolites, is responsible for inhibition of axonal growth.

5.4 Discussion

The initial goal was to investigate the role of GSLs in axonal growth. A comparison of the effects of two inhibitors of the endogenous synthesis of GSLs (FB_1 and PPMP) suggests that newly synthesized GSLs are not required for normal axonal elongation, rather the lipid second messenger ceramide negatively regulates axonal growth.

Newly-synthesized GSLs are not essential for axonal growth

Both FB_1 and *threo*-PPMP inhibited the incorporation of [^{3}H]palmitate into several gangliosides in rat sympathetic neurons (Fig. 5.2 and Table 5.1) indicating that these agents inhibit GSL synthesis. Consequently, since only *threo*-PPMP, but not FB_1, impaired axonal growth, newly-synthesized GSLs are not essential for axonal growth. This finding is in accordance with a study by Li and Ladisch (40) in which PDMP treatment reduced ganglioside synthesis by greater than 90% but did not block neurite formation induced by retinoic acid in a neuroblastoma cell line. Similarly, Schwarz *et al.* (20) reported a dramatic decrease in the amount of cell surface gangliosides as early as 24 h after treatment with FB_1 or PDMP in hippocampal neurons. However, no effect on axonal growth was observed at this time or even 48 h after the addition of inhibitor; inhibition of GSL synthesis did not affect the formation of the parent axon during its emergence from the cell body. In hippocampal neurons, the most significant effect of inhibition of GSL synthesis was impairment of the formation or stabilization of collateral axonal branches (20). Furuya *et al.* (21) observed aberrant growth of dendrites of Purkinje cells depleted of membrane GSLs by FB_1 treatment,

and FB_1 treatment resulted in less complex patterns of dendritic arborization, suggesting a role of GSLs in neurite branching. However, no differences in axonal outgrowth were observed between treated and untreated cultures. Unfortunately, the growth assay used in this study does not monitor branching. Nor do experiments presented in this Chapter exclude the possibility that chronic inhibition of GSL biosynthesis, leading to the depletion of cellular GSLs, would eventually affect axonal growth.

Ceramide negatively regulates axonal growth

Several lines of evidence from this study imply that the distinct effects of FB_1 and PPMP on axonal growth relate to their differential effects on ceramide metabolism. Treatment of rat sympathetic neurons with FB_1 reduced the incorporation of [^{3}H]palmitate into ceramide (Fig. 5.5A) whereas the opposite effect (increased incorporation of [^{3}H]palmitate into ceramide) was observed in *threo*-PPMP-treated cells (Fig. 5.5B). Furthermore, pretreatment of neurons with FB_1 prevented the inhibitory effect of *threo*-PPMP on axonal growth (Fig. 5.6). In addition, exogenously added cell-permeant ceramide mimicked the effect of *threo*-PPMP on axonal growth (Fig. 5.7). In light of the potent biological effects of ceramide on cells (reviewed in 6 and 7), these results imply that ceramide is involved in the regulation of axonal growth.

A signal transduction pathway initiated by SM hydrolysis and leading to the generation of the second messenger ceramide has been proposed (6, 41). Candidate cellular targets for ceramide include a ceramide-activated protein kinase (42), a ceramide-activated protein phosphatase (43, 44) and protein kinase Cζ (45). A number of extracellular agents, including tumor necrosis factor-α (46-48), interferon-γ (46) and interleukin-1 (49, 50), activate SM hydrolysis and generate ceramide upon receptor binding. The involvement of this pathway in neuronal signaling is also suggested by the finding that NGF (51), as well as other neurotrophins (52), rapidly activate SM hydrolysis in cell lines expressing the p75 neurotrophin receptor, $p75^{NTR}$. In actively dividing cells, a growing body of data suggests a role for ceramide in growth suppression and apoptosis (6, 7). The role of growth factor-induced SM catabolism in post-mitotic neurons, however, has not been elucidated. Our study suggests that ceramide negatively regulates axonal growth. It is clear from studies with compartmented rat sympathetic neurons that regulation of axonal growth in response to NGF involves mechanisms localized to the site of NGF application and does not directly involve mechanisms within the cell body (reviewed in 53). It would be logical, therefore, that factors involved in regulation of axonal growth would act at the site of NGF application. Ceramide is a candidate factor since it inhibits axonal growth of compartmented sympathetic neurons only when applied to distal axons but not when applied to cell bodies (Fig. 5.7A). The specificity of the effect of ceramide on axonal growth is demonstrated by the lack of any effect of dihydroceramide, which

differs from ceramide only in the absence of a 4,5 *trans*-double bond, on axonal growth (Fig. 5.7B).

Ito and Horigome (54) have recently demonstrated that cell-permeant ceramide delays the onset of programmed cell death induced by NGF deprivation in cultured rat sympathetic neurons. The authors suggest that NGF may suppress programmed cell death by activation of SM hydrolysis via $p75^{NTR}$. Thus ceramide might be involved in both survival and growth of axons of sympathetic neurons. A role for the $p75^{NTR}$ receptor in neuronal survival and axon growth has already been suggested by studies using p75-deficient mice (55-57). NGF-responsive neurons might regulate axonal growth via the cross-talk between signaling pathways induced by trkA and $p75^{NTR}$. A mechanism could be envisioned in which under conditions favoring growth, trkA signaling is active and suppresses the ability of $p75^{NTR}$ to mediate SM hydrolysis to ceramide, thus promoting axonal growth. Under conditions where growth arrest and retraction of axons are required, the activity of trkA might be inhibited leading to the induction of SM hydrolysis via $p75^{NTR}$ and rapid arrest of axonal growth. One model of neuronal pathfinding suggests growth-promoting and growth-inhibitory signals are interpreted in the growth cone and translated into a directed growth response (58, 59). The local generation of ceramide may be one inhibitory signal regulating neuronal pathfinding.

The mechanism by which ceramide suppresses axonal growth remains to be determined. Possibly, downstream targets of ceramide, such as ceramide-activated protein kinase (42), ceramide-activated protein phosphatase (43, 44) or protein kinase Cζ (45) are activated. The inability of dioctanoylglycerol, a cell-permeant diacylglycerol, to reverse the inhibition of axonal growth by ceramide highlights important differences between mechanisms involved in the regulation of axonal growth and those governing cell viability and induction of apoptosis since the induction of apoptosis by ceramide has been shown to be reversed by the simultaneous addition of diacylglycerol or phorbol esters (6, 37).

Ceramide can be converted to a number of putative bioactive lipids, such as sphingosine and ceramide-1-phosphate. It is, therefore, possible that a metabolite of ceramide might be responsible for the effects of ceramide on axonal growth. Indeed, sphingosine suppresses NGF-induced neurite sprouting in PC12 cells (60). Similarly, treatment of distal axons, but not cell bodies, of compartmented sympathetic neurons with sphingosine inhibits axonal growth (61). No effect of ceramide on the axonal growth of sympathetic neurons was observed in the latter study since long-chain, non-cell permeant ceramides were used. Addition of exogenous sphingosine rapidly elevates cellular ceramide levels in A431 cells and Neuro2a cells (62, 63), suggesting that some effects of sphingosine may be mediated by its conversion to ceramide. The reverse reaction, the metabolism of exogenous short-chain ceramide to sphingosine, has also been demonstrated in Chinese hamster ovary and Madin-Darby canine

kidney cells (38, 39). The time course and quantity of sphingosine generation from exogenous short-chain ceramide, however, suggest that sphingosine is unlikely to mediate the rapid effects of ceramide on cells (36). Ceramide-1-phosphate is generated via the phosphorylation of ceramide by ceramide kinase (64) and the activity of a calcium-dependent ceramide kinase has been detected in rat brain synaptic vesicles (65). It is, however, unlikely that ceramide-1-phosphate mediates the effects of ceramide on axonal growth since apparently only ceramide generated from SM hydrolysis, not *de novo* synthesized ceramide, serves as a substrate for ceramide kinase (66). Moreover, exogenous C_2-ceramide is poorly metabolized into C_2-ceramide phosphate (36). In axons of rat sympathetic neurons only 2.7% of cell-associated NBD-C_6-ceramide was converted into ceramide phosphate (see Section 5.3.5 above). It is, therefore, concluded that ceramide itself mediates the inhibition of the growth of axons of sympathetic neurons.

In summary, the results presented in this Chapter demonstrate that newly-synthesized GSLs are not essential for axonal growth. More importantly, these studies support and strengthen the idea that axonal growth is locally regulated within the axon itself and strongly suggest that the lipid second messenger ceramide negatively regulates axonal growth.

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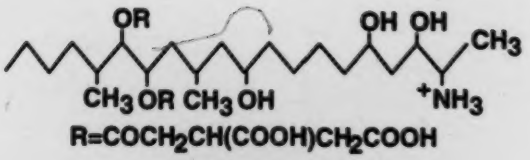
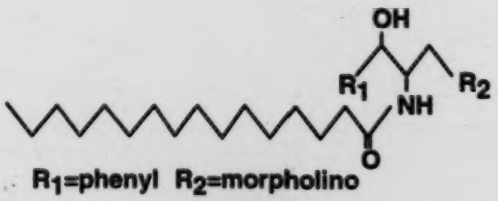


Fumonisin B_1



PPMP

Fig. 5.1 Structures of Fumonisin B_1 and PPMP

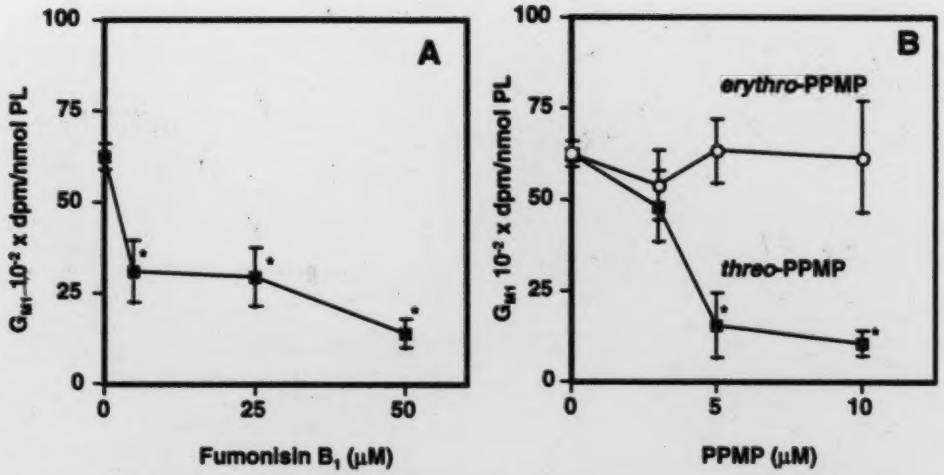


Fig. 5.2 **FB_1 and *threo*-PPMP reduce the incorporation of [^{3}H]palmitate into ganglioside G_{M1}**

Sympathetic neurons were grown for 11 days in 24-well dishes, then treated for 3 days with the indicated concentrations of FB_1 (A), *threo*-PPMP (squares, B) or *erythro*-PPMP (circles, B) for 3 days. Incubation was continued for an additional 1 day in the presence of [^{3}H]palmitate (10 μCi/ml). Cells were collected and lipids extracted and separated by thin-layer chromatography. Data are dpm in G_{M1} per nmol of total lipid phosphorus (PL) and are averages ± S.D. of four individual cultures. The experiment was repeated three times with similar results. Significantly different from control (no inhibitor): $^*p < 0.05$.

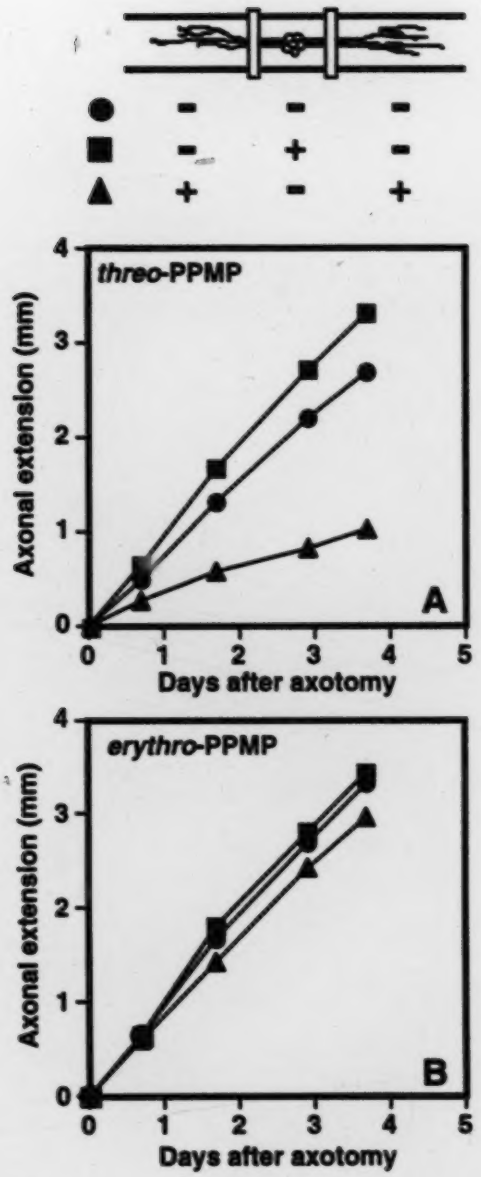
Table 5.1 **FB_1 and *threo*-PPMP inhibit [^{3}H]palmitate incorporation into gangliosides**

Neurons (11 days old) cultured in 24-well dishes were incubated in the presence or absence of 25 μM FB_1 or 5 μM *threo*- or *erythro*-PPMP for 4 days. [^{3}H]Palmitate (10 μCi/ml) was present during the last 24 h of incubation. The cells were collected and lipids separated. Data are dpm recovered in each ganglioside (G_{M1}, G_{M3} or G_{T1b}) per nmol of total lipid phosphorus and are averages ± S.D. for four individual cultures. The experiment was repeated three times with similar results. Significantly different from control (no inhibitor): $^*P<0.05$.

Treatment	G_{M1}	G_{M3} (dpm/nmol PL)	G_{T1b}
none (Control)	6020 ± 332	2142 ± 128	1098 ± 158
5 μM *erythro*-PPMP	6304 ± 825	2038 ± 298	1035 ± 142
5 μM *threo*-PPMP	1535* ± 904	392* ± 109	104* ± 35
25 μM Fumonisin B_1	2913* ± 794	759* ± 154	285* ± 60

Fig. 5.3 ***threo*-PPMP applied to distal axons inhibits axonal growth**

Neurons were grown as compartmented cultures for 14 days and then incubated for 3 days without (-) or with (+) 5 μM *threo*-PPMP (A) or *erythro*-PPMP (B) in either the center compartment or side compartments. Neurons were subsequently neuritotomized and allowed to regenerate under the same conditions. Axonal extension was measured at indicated times. Results are means ± S.E. (error bars contained within *symbols*) of measurements from 80 to 100 tracks for each treatment. The experiment was repeated three times with similar results.


- - -
- + -
+ - +
threo-PPMP
Axonal extension (mm)
Days after axotomy
A
erythro-PPMP
Axonal extension (mm)
Days after axotomy
B

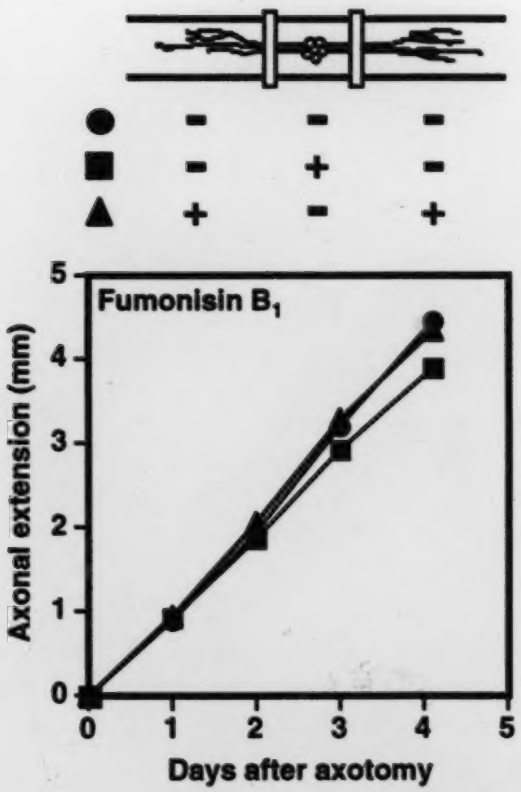
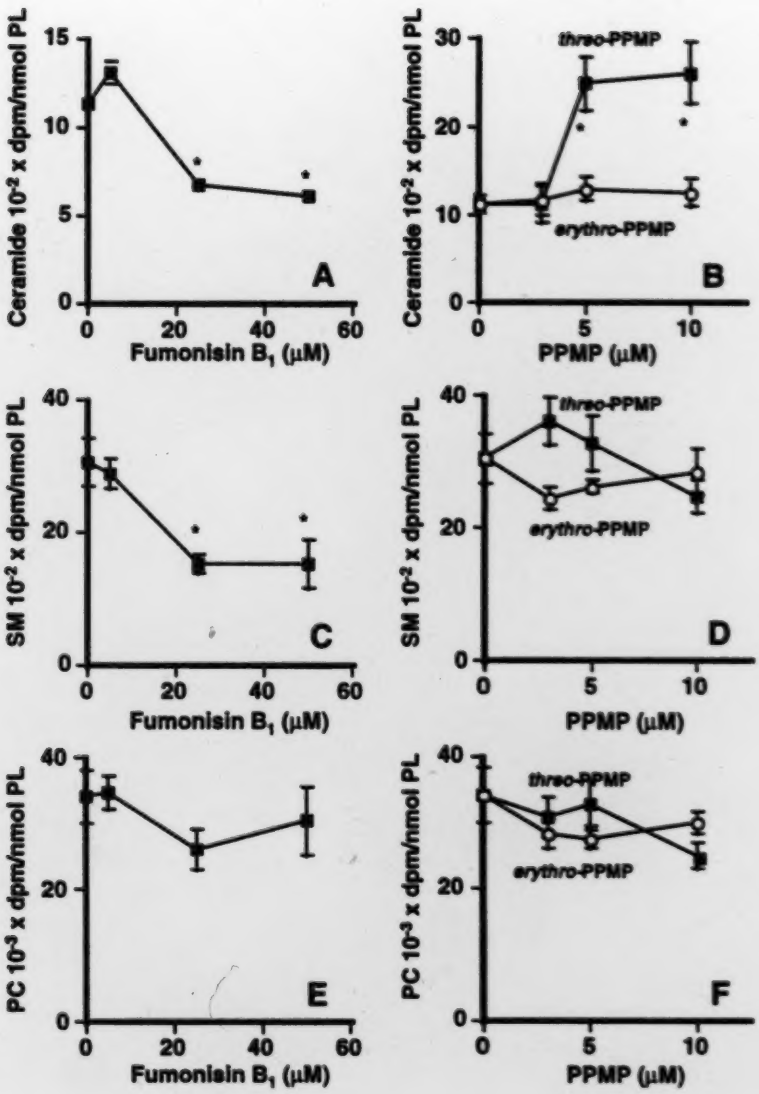


Fig. 5.4 **FB_1 does not inhibit axonal growth of rat sympathetic neurons**

Neurons cultured for 13 days in compartmented dishes were incubated for 3 days without (-) or with (+) 25 μM FB_1 in either the cell body-containing compartment or distal axon-containing compartments. Cultures were neuritotomized and allowed to regenerate under pre-axotomy conditions. Axonal extension was measured at indicated times. Results are means ± S.E. (error bars contained within *symbols*) of measurements from 80 to 100 tracks for each treatment. The experiment was repeated three times with similar results.

Fig. 5.5 ***threo*-PPMP increases, whereas FB_1 decreases the incorporation of [^{3}H]palmitate into ceramide**

See Fig. 5.3 for experimental details. A and B, ceramide; C and D, SM; E and F, PC. B, D and F, *threo*-PPMP = squares, *erythro*-PPMP = circles. Significantly different from control (no inhibitor): $^*p < 0.05$.


Ceramide 10⁻² x dpm/nmol PL
Fumonisin B1 (μM)
A
Ceramide 10⁻² x dpm/nmol PL
threo-PPMP
erythro-PPMP
PPMP (μM)
B
SM 10⁻² x dpm/nmol PL
Fumonisin B1 (μM)
C
SM 10⁻² x dpm/nmol PL
threo-PPMP
erythro-PPMP
PPMP (μM)
D
PC 10⁻³ x dpm/nmol PL
Fumonisin B1 (μM)
E
PC 10⁻³ x dpm/nmol PL
threo-PPMP
erythro-PPMP
PPMP (μM)
F

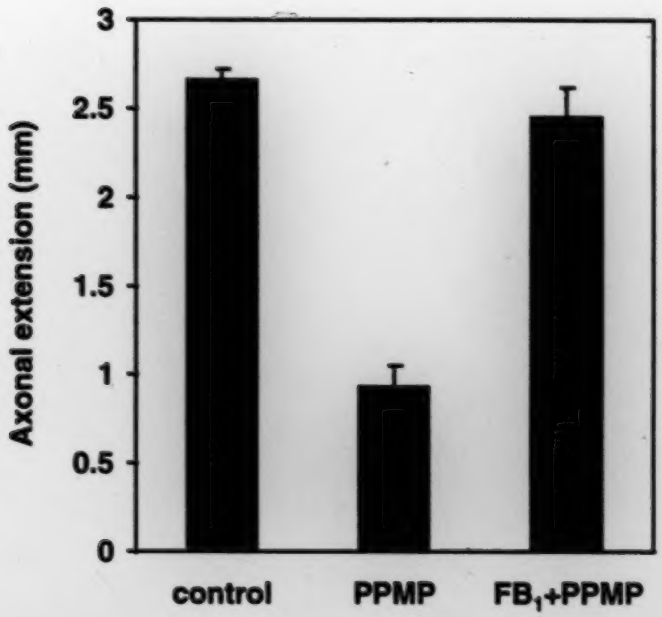
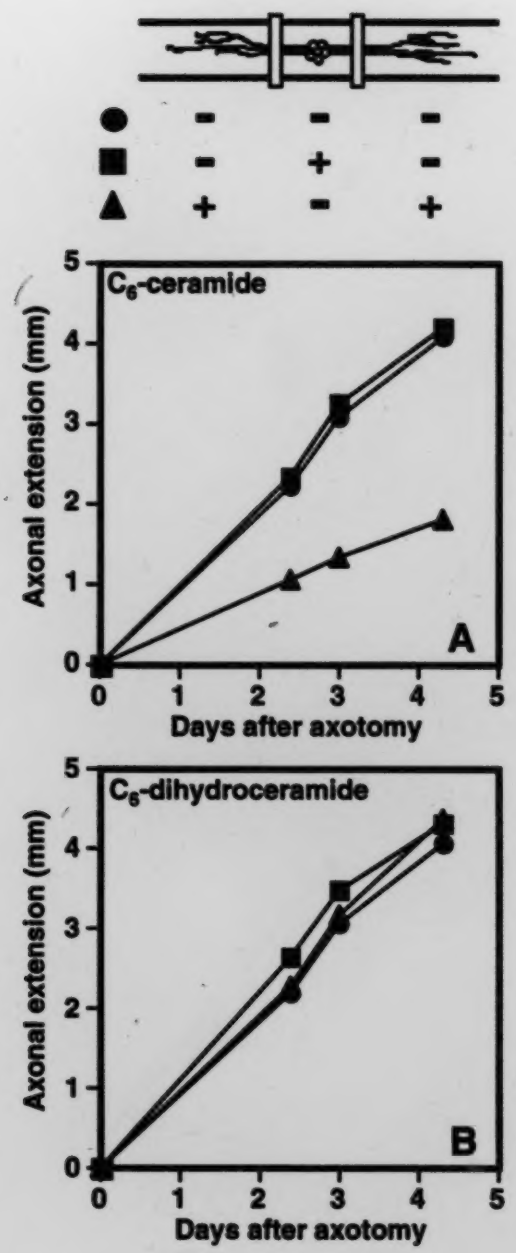


Fig. 5.6 **Pretreatment with FB_1 prevents *threo*-PPMP inhibition of axonal growth**

Neurons cultured for 13 days in compartmented dishes were either left untreated for 7 days, left untreated for 4 days and then treated with 5 μM *threo*-PPMP for 3 days or treated with 25 μM FB_1 for 4 days and then treated with 5 μM *threo*-PPMP for 3 days. At that time, cultures were axotomized and allowed to regenerate under the same conditions as before. Axonal extension was measured 3 days after axotomy. Results are means ± S.E. of measurements from 60 to 100 tracks for each treatment.

Fig. 5.7 **C_6-ceramide inhibits axonal growth when applied to distal axons but not when applied to cell bodies**

Neurons cultured for 13 days in compartmented dishes were either untreated (-) or treated (+) with 10 μM C_6-ceramide (A) or 10 μM C_6-dihydroceramide (B) for 3 days in either the cell body-containing compartment or distal axon-containing compartments. At that time, cultures were neuritotomized and allowed to regenerate under the same conditions as before. Axonal extension was measured at indicated times. Results are means ± S.E. (error bars contained within *symbols*) of measurements from 80 to 100 tracks for each treatment. The experiment was repeated three times with similar results.


- - -
- + -
+ - +
C_6-ceramide
Axonal extension (mm)
Days after axotomy
A
C_6-dihydroceramide
Axonal extension (mm)
Days after axotomy
B

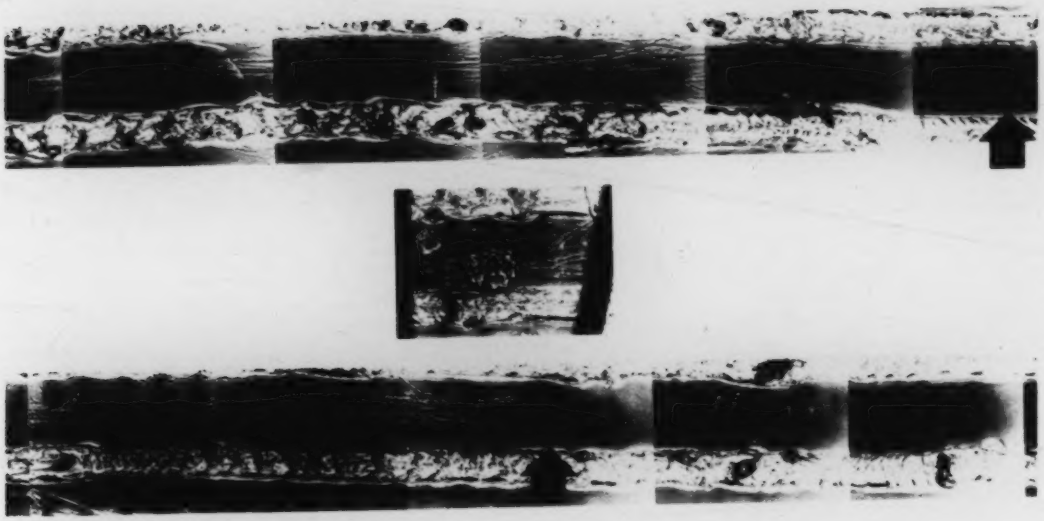


Fig. 5.8 **Morphology of distal axons treated with C_6-ceramide**

Neurons cultured for 13 days in compartmented dishes were axotomized and allowed to regenerate for 1 day, after which the *right side* compartment was supplied with 10 μM C_6-ceramide. The *left side* compartment was untreated and acted as a control. Shown is a continuous sequence of phase-contrast photomicrographs of the neurons 24 h after treatment of the *right side* compartment with C_6-ceramide. *Top* and *bottom* panels show axons along a single track in the left (*top panel*) and right (*bottom panel*) compartments. The *middle panel* shows cells bodies and proximal neurites in the center compartment of the same track. Scratches bordering the track are visible at the *top* and *bottom* of each panel, and the silicone grease is visible at the sides. Arrows indicate the tip of the longest axon in the *left* and *right* compartments.

CHAPTER 6

On the Mechanism of Ceramide-Mediated Inhibition of Axonal Growth of Rat Sympathetic Neurons

6.1 INTRODUCTION

Agonist-induced activation of sphingomyelinase resulting in the generation of ceramide has been implicated as a signaling pathway involved in the regulation of cellular proliferation, differentiation and apoptosis (1, 2). Studies in Chapter 5 suggest that ceramide might also be involved in the regulation of the axonal growth of neurons. Elevation of ceramide levels within distal axons, but not within cell bodies, of rat sympathetic neurons inhibited axonal growth, suggesting that the local generation of ceramide might be an inhibitory signal regulating axonal growth (Chapter 5).

The mechanisms which regulate axonal growth are complex and have not been completely elucidated. As mentioned previously, evidence from work with compartmented cultures of rat sympathetic neurons indicates that axonal growth in response to NGF involves mechanisms localized to the site of NGF application and does not directly involve mechanisms within the cell body (3). Association of NGF with trkA, a receptor tyrosine kinase, initiates a signal transduction cascade which ultimately results in the promotion of axonal growth and neuronal survival (reviewed in 4 and 5). Treatments which interfere with signaling from trkA, impair neuronal growth and survival (6-8).

Another factor involved in the regulation of axonal growth is the axonal synthesis of membrane lipids. Membrane lipid biosynthesis has been shown to occur locally

within axons (9, 10), as well as in cell bodies, and this axonal lipid synthesis plays a key role in membrane biogenesis during axonal growth and regeneration (11). Treatments that inhibit axonal synthesis of PC, the predominant phospholipid of eukaryotic membranes, inhibit axonal growth (11, 12).

The goal of studies in this Chapter was to identify the mechanisms involved in ceramide-mediated inhibition of axonal growth. The experimental approach was to determine the effect of ceramide on cellular processes known to be involved in regulating axonal growth, such as NGF signaling via trkA receptors and axonal lipid synthesis.

6.2 Materials and Methods

[*methyl*-^{14}C]SM (45 mCi/mmol) was purchased from Amersham Canada, Oakville, ON, Canada. All other reagents and chemicals used are listed in the Material and Methods sections of Chapters 2, 3 and 5.

Preparation of neuronal cultures and measurement of axonal extension

The general procedures for preparation of neuron cultures were described in Chapter 2 Section 2.2 and Chapter 3 Section 3.2. Left-plated compartmented cultures used in studies described in this Chapter were constructed essentially as described in Chapter 3 Section 3.2 with the exception that silicone grease was applied in the left compartment of cultures to create a small pocket of restricted area used to plate the

dissociated neurons (Fig. 6.1). Neurons were plated at a density of 0.6 to 0.8 ganglia/dish in the left compartment of compartmented culture dishes (Fig. 6.1). After 2 weeks of growth, the left side compartment contains cell bodies and proximal neurites, the center compartment contains axons and the right side compartment contains axons and axon terminals. For the first 6 days of growth, medium supplied to the left cell body-containing compartment is supplemented with 2.5% rat serum, 1 mg/ml ascorbic acid, 10 μM cytosine arabinoside (to prevent the growth of non-neuronal cells) and 10 ng/ml NGF. Medium supplied to the center compartment contained 20 ng/ml NGF and medium supplied to the right side compartment contained 100 ng/ml NGF. These conditions promote the growth of axons into the right side compartment. After 6 days, cytosine arabinoside treatment is discontinued in the left side and NGF is confined to the right side compartment. Thus, axonal growth is restricted to the right side compartment. Culture medium is routinely changed every 4-6 days, thereafter.

Axotomy of left-plated cultures was performed as described in Chapter 5 Section 5.2 with the exception that only axons in the right side compartment were removed. Axonal growth was determined by measurement of elongation of axons in this compartment (right side compartment).

C_6-NBD-ceramide transport

C_6-NBD-ceramide (20 μM) was supplied to either the center or side compartments of 13-day-old center-plated compartmented cultures of rat sympathetic neurons. After 24 h the medium was aspirated, cellular material from the center and side compartments was harvested separately and the lipids were extracted (13). Aliquots of the organic phase were dried and used to determine total lipid phosphorus and to measure fluorescence. Fluorescence was quantitated using a Hitachi F-2000 fluorimeter (excitation wavelength = 464 nm, emission wavelength = 532 nm).

Sphingomyelinase activity

Determination of neutral and acid sphingomyelinase activity was performed essentially as described in published protocols (14-16). The assay was optimized for detection of SMase activity from cultured rat sympathetic neurons.

Preparation of substrate

Substrate was prepared by resuspending 60 pmol of [*methyl*-^{14}C]SM (45 mCi/mmol) and 900 pmol of unlabeled SM in 25 μl of buffer per assay. The substrate was resuspended in a buffer containing 0.2 M Tris-HCl (pH 7.5), 0.1% Triton X-100, 10 mM $MgCl_2$ for determination of neutral SMase activity or in buffer 0.2 M sodium acetate (pH 5.0), 0.1 % Triton X-100 for determination of acid SMase activity.

Neutral or Acid SMase assay

Cells were washed extensively with ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) and collected in ice-cold harvest buffer which consisted of 10

mM Tris (pH 7.4), 0.1% Triton X-100 and protease inhibitors (0.1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 0.1 mM PMSF). A sonicate was prepared and centrifuged for 5 min at 800xg. An aliquot of the sonicate containing 20-30 μg of total protein in a volume of 25 μl (volume adjusted with substrate resuspension buffer) was used in a typical assay. The enzymatic reaction was initiated by addition of 25 μl of substrate and incubation at 37°C. After 30 min the reaction was terminated by addition of $CHCl_3/CH_3OH$ 2:1 and H_2O to obtain a final ratio of $CHCl_3/CH_3OH/H_2O$ of 2:1:1 (17). The phases were separated and the amount of radioactivity in the aqueous phase, containing the [^{14}C]phosphocholine released by hydrolysis, was determined by liquid scintillation counting.

Metabolic labeling of neurons with [^{3}H]choline

Axons in the side compartments of 13-day old compartmented cultures of neurons were metabolically labeled with [^{3}H]choline (10 μCi/ml) for 6 h in the absence or presence of 10 or 20 μM C_6-ceramide. Cultures were washed three times with ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) and the cellular material in the side compartments harvested in CH_3OH/H_2O 1:1. Lipids were extracted, separated by thin-layer chromatography and the incorporation of [^{3}H]choline into PC was determined by scraping the band corresponding to PC followed by liquid scintillation counting as described in Chapter 2 Section 2.2.

Detection of protein tyrosine phosphorylation

Center-plated compartmented cultures of neurons grown for 10 days in medium containing 100 ng/ml NGF in the side compartments were washed twice with NGF-free medium and incubated for 3 days with medium containing 20 ng/ml NGF. On the day of the experiment, cultures were washed twice with NGF-free medium and incubated for 6 h in the absence of growth factors or serum. Since sympathetic neurons require NGF for growth and survival in culture, these steps were taken in order to obtain sufficiently dense cultures for experimentation and to reduce the basal level of protein tyrosine phosphorylation in the cultures by progressively reducing the concentration of NGF in the medium. At this time, cultures were given medium containing either 200 ng/ml NGF, 20 ng/ml NGF, 25 μM C_6-ceramide, or both NGF and ceramide, as indicated, to the side distal axon-containing compartments. Extracts of the cellular material from the side compartments were prepared at various times after treatment. Cultures were rapidly washed three times with ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 1 mM sodium orthovanadate. Axons were collected in Tris-buffered saline containing 0.5% SDS, 1 mM sodium orthovanadate and protease inhibitors. Equal amounts of protein were subjected to 7.5% polyacrylamide gels followed by transfer onto nitrocellulose and Western blotting with antiphosphotyrosine antibody 4G10 (UBI, Lake Placid, NY) and detection by enhanced chemiluminescence (ECL, Amersham). The same procedure was followed in experiments using mass cultures of neurons.

Other procedures

Protein amount was determined using the micro version of the bicinchoninic acid procedure (Pierce kit), using bovine serum albumin as the standard. The phospholipid content of cells was measured by lipid phosphorous determination (18).

6.3 RESULTS

6.3.1 The inhibitory effect of ceramide on axonal growth is localized to axon terminals

As described in Chapter 5, inhibition of axonal growth was observed only in response to elevation of ceramide within distal axons, but not within cell bodies and proximal neurites. To localize further the inhibitory effect of ceramide on axonal growth, left-plated compartmented cultures of rat sympathetic neurons were used, in which dissociated neurons were initially plated in the left side compartment of 3-compartment dishes. In 2 week-old left-plated cultures, growth is restricted to the right side compartment since NGF is only supplied to this compartment. The right side compartment of left-plated cultures, therefore, contains distal axons and axon terminals, the left side compartment contains the cell bodies and proximal neurites and the center compartment solely contains axons (Fig. 6.1). Using these cultures, it was determined whether C_6-ceramide could inhibit distal axon growth when given to axons alone or whether ceramide only inhibits growth when given to growing axon terminals. Cultures grown for 2 weeks were axotomized and allowed to regenerate for 3 days under normal culturing conditions. At this time, axonal extension was

measured and cultures were then either left untreated, treated with 10 μM C_6-ceramide in the right side compartment alone or treated with 30 μM C_6-ceramide in the center compartment alone. Axonal extension was measured 24 h later. The axons of untreated (control) cultures elongated 0.63 ± 0.09 mm in this period (Fig. 6.2). As expected, the axons of cultures treated with 10 μM C_6-ceramide in the right side compartment alone did not elongate and retracted by 0.74 ± 0.08 mm (Fig. 6.2). Surprisingly, cultures treated with 30 μM C_6-ceramide in the center compartment alone elongated to the same extent as untreated (control) cultures (0.68 ± 0.08 mm) (Fig. 6.2). These results show that the inhibitory effect of ceramide on axonal growth is localized to the growing region of a neuron, the axon terminals, which is also the site of NGF application.

6.3.2 Anterograde axonal transport of ceramide is inefficient

One possible explanation for the lack of effect of ceramide on axonal growth when supplied to cell bodies or axons is that ceramide is not anterogradely transported to its site of action, the distal ends of growing axons. In order to address this possibility, the axonal transport of a fluorescent, short-chain analog of ceramide, C_6-NBD-ceramide was examined. Studies in Chapter 5 (Section 5.3.5) demonstrated that C_6-NBD-ceramide exhibits the same axonal growth inhibitory properties as C_6-ceramide. C_6-NBD-ceramide (20 μM) was supplied to either the center or side compartments of center-plated compartmented cultures of rat sympathetic neurons. After an overnight incubation, cellular material from the center and side compartments was harvested

separately, the lipids extracted and the concentration of fluorescence in each compartment was determined. In cultures given C_6-NBD-ceramide to the center compartment alone, the concentration of fluorescence in the cellular material collected from the center compartment was 3.13 units/µmol total lipid phosphorus and the concentration in cellular material from the side compartments was 0.44 units/µmol total lipid phosphorus. In cultures supplied with C_6-NBD-ceramide to the side compartments alone, the concentration of fluorescence in cellular material collected from the center compartments was 1.79 units/µmol total lipid phosphorus and 8.53 units/µmol total lipid phosphorus in cellular material from the side compartments. These results suggest that axonal transport of ceramide is inefficient. Therefore, C_6-ceramide supplied to cell bodies and proximal neurites might not inhibit distal axon growth because inhibitory concentrations of ceramide are not achieved within the distal ends of the axons, the site of action of ceramide.

6.3.3 Acid SMase localizes to cell bodies and neutral SMase is enriched in axons and axon terminals of rat sympathetic neurons

Agonist-induced generation of ceramide involves the activation of cellular SMases which have either acid or neutral pH optima (see Chapter 4 Section 4.3.2). The localization of acid and Mg^{2+}-dependent neutral SMase activity in center-plated compartmented cultures of neurons was investigated. Cellular material from the center cell-body-containing compartment and side distal axon-containing compartments was harvested and assayed *in vitro* for acid and Mg^{2+}-dependent

neutral SMase activity. A distinct localization of acid and neutral SMase activity was observed (Fig. 6.3). Acid SMase activity was almost exclusively localized to the center compartment, and consequently to the cell bodies and proximal neurites of rat sympathetic neurons (Fig. 6.3). Neutral Mg^{2+}-dependent SMase activity, however, was enriched in the side distal axon-containing compartments (Fig. 6.3). The distribution of SMase activity within rat sympathetic neurons might have important implications for agonist-induced SM hydrolysis. Ceramide involved in axon growth inhibition could be generated by activation of Mg^{2+}-dependent neutral SMase.

6.3.4 Ceramide does not inhibit axonal PC synthesis

Previous studies have demonstrated that PC is synthesized within the axons, as well as cell bodies, of neurons (9, 10) and that the local synthesis of PC within axons is critical for axonal growth and regeneration (11). Inhibition of PC synthesis within axons due to choline deficient conditions (11) or due to treatment with metabolic inhibitors (12), severely impairs axonal elongation. The possibility that ceramide was inhibiting axon growth via inhibition of axonal PC synthesis was therefore examined. Sympathetic neurons were metabolically labeled with [^{3}H]choline for 6 h in the absence or presence of 10 or 20 μM C_6-ceramide. Incorporation of [^{3}H]choline into PC in distal axons was increased slightly by C_6-ceramide treatment (Fig. 6.4), ruling out the possibility that ceramide was inhibiting axonal growth via inhibition of PC synthesis.

6.3.5 Ceramide attenuates protein tyrosine phosphorylation induced by NGF

NGF promotes the survival and axonal growth of cultured rat sympathetic neurons (4, 5, 19). Association of NGF with trkA, a receptor tyrosine kinase, initiates a cascade of protein tyrosine phosphorylation and signaling events which ultimately results in the stimulation of axonal growth and survival (3-5). It is possible that ceramide might be inhibiting axonal growth by interfering with signaling cascades initiated by the association of NGF with its receptor, trkA. The effect of cell-permeant ceramides on protein tyrosine phosphorylation induced by NGF was therefore examined. Compartment cultures of neurons grown for 10 days in medium containing 100 ng/ml NGF in the side compartments were washed extensively with NGF-free medium and incubated for 3 days with medium containing 20 ng/ml NGF in the side compartments. On the day of the experiment, cultures were again washed extensively with NGF-free medium and incubated for 6 h in the absence of growth factors or serum. These steps were taken in order to obtain sufficiently dense cultures for experimentation and to reduce the basal level of protein tyrosine phosphorylation in the cultures by progressively reducing the concentration of NGF in the medium. Cultures were given medium containing 200 ng/ml NGF with or without 25 μM C_6-ceramide to the side distal axon-containing compartments. Extracts of the cellular material from the side compartments were prepared at various times after treatment and equal amounts of protein were subjected to 7.5% SDS-polyacrylamide gels followed by Western blot analysis with antiphosphotyrosine antibody (4G10). A basal level of protein tyrosine phosphorylation was observed in untreated cultures (time 0)

(Fig. 6.5A). NGF treatment rapidly induced the tyrosine phosphorylation of a number of proteins of different molecular mass (Fig. 6.5A), as has been previously reported (6, 20). The 140 kDa tyrosine phosphorylated protein has previously been identified as the NGF receptor trkA, which autophosphorylates on tyrosine residues upon ligand binding (6, 19). No differences in the pattern or magnitude of protein tyrosine phosphorylation induced by 200 ng/ml NGF in the absence or presence of ceramide was observed (Fig. 6.5A). An effect of ceramide on NGF-induced protein tyrosine phosphorylation might not be detected in the presence of saturating concentrations of NGF. The above experiment was, therefore, repeated using a sub-maximal dose of NGF, 20 ng/ml. A less robust burst of protein tyrosine phosphorylation was observed using 20 ng/ml NGF (Fig. 6.5B). Although the pattern of protein tyrosine phosphorylation did not change in the presence of ceramide, the time course was different (Fig. 6.5B). Protein tyrosine phosphorylation in the presence of ceramide was less pronounced after 1 and 2 h when compared to phosphorylation induced by 20 ng/ml NGF in the absence of ceramide (Fig. 6.5B). These results suggest that ceramide might attenuate NGF signaling in distal axons of sympathetic neurons. Ceramide treatment alone did not induce the tyrosine phosphorylation of any proteins (Fig. 6.6). Although an increased tyrosine phosphorylation of the 140 kDa band is apparent after 60 and 120 min of C_6-ceramide treatment in the Western blot shown in Fig. 6.6, this result was not obtained in replicate experiments.

6.4 Discussion

The goal of studies described in this Chapter was to begin to define the mechanism(s) by which ceramide inhibits axonal growth. The data show that the inhibitory effect of ceramide on axonal growth is restricted to axon terminals. Elevation of ceramide within cell bodies or axons does not inhibit axonal growth probably due to the inefficient axonal transport of ceramide. The localization of acid SMase activity to cell bodies and neutral SMase activity to axons and axon terminals of sympathetic neurons suggests that elevation of ceramide within distal axons might be produced by the activation of neutral SMase activity. Several potential mechanisms by which ceramide might inhibit axonal growth were investigated including effects on PC synthesis and tyrosine phosphorylation of cellular proteins. Treatment of neurons with cell-permeant ceramide did not inhibit axonal PC synthesis, which is required for axonal growth and regeneration (11). Although ceramide itself did not induce the tyrosine phosphorylation of any proteins, ceramide treatment attenuated protein tyrosine phosphorylation induced by less than saturating concentration of NGF in distal axons.

When the center compartments of compartmented cultures of neurons were supplied with C_6-NBD-ceramide, fluorescence was observed in distal axons in the side compartments (see Section 6.3 above). Previous studies have demonstrated that fluorescence is rapidly observed in the plasma membrane and internal membranes of fibroblasts given C_6-NBD-ceramide (21-23). Fluorescent labeling of internal

membranes was observed even under conditions where endocytosis was blocked (low temperature) (20-22), suggesting that ceramide incorporated in the outer leaflet of the plasma membrane can spontaneously flip to the inner leaflet and enter cells. Two explanations could account for a lack of effect of ceramide given to cell bodies and proximal neurites on distal axon growth. The concentration of fluorescence measured in distal axons of compartmented cultures given C_6-NBD-ceramide to the center compartment (0.44 units/μmol total lipid phosphorus) was much less than the concentration of fluorescence in distal axons given C_6-NBD-ceramide directly (8.53 units/μmol total lipid phosphorus). Therefore, C_6-ceramide supplied to cell bodies and proximal neurites might not inhibit distal axon growth because axonal transport of ceramide is inefficient and inhibitory concentrations of ceramide are not attained within the distal ends of axons, the site of action of ceramide (Fig. 6.2). In Chapter 5, it was shown that C_6-NBD-ceramide is poorly metabolized by sympathetic neurons. It is possible, however, that some or all of the fluorescence that was axonally transported represents a product of ceramide metabolism such as SM or glucosylceramide. An alternative explanation for the restricted site of action of ceramide on axonal growth is that a distinct pool of ceramide might exist in axon terminals which is involved in the regulation of axonal growth. Ceramide transported from cell bodies and proximal neurites to the axon terminals might not mix with this pool and therefore has no effect on growth. Evidence is accumulating which supports the existence of "signaling" pools of SM and ceramide within cells (24-27 and see Chapter 4 Section 4.4.2.1).

Ceramide can be generated from the hydrolysis of SM as a biological response to several extracellular agents and insults (see Chapter 4 Section 4.4.2.1). The breakdown of SM to ceramide is catalyzed by cellular SMases. Several SMases have been characterized with neutral and acidic pH optima (see Chapter 4 Section 4.3.2 and 4.4.2.1). The restricted localization of acid SMase activity to cell bodies and enrichment of Mg^{2+}-dependent neutral SMase activity in axons and axon terminals of rat sympathetic neurons (Fig. 6.3) has important implications for agonist-induced SM hydrolysis. Both neutral and acid SMase have been postulated to be involved in the generation of ceramide as a signaling event (1, 2). Since axonal growth is inhibited by ceramide only when ceramide levels are elevated within distal axons but not within cell bodies or proximal neurites, we speculate that ceramide involved in axonal growth inhibition can be generated by activation of a Mg^{2+}-dependent neutral SMase. Acid SMase is a lysosomal enzyme and the localization of its activity to the cell bodies of sympathetic neurons correlates with reports which show that lysosomes and late endosomes are concentrated near and within the cell bodies of neurons (reviewed in 28, 29).

Several potential mechanisms by which ceramide inhibits axonal growth were examined. The local synthesis of PC within axons is essential for maintenance of the supply of membrane required during axonal growth and regeneration. In fibroblast cell lines, PC synthesis was markedly inhibited by treatment with cell-permeant

analogs of ceramide (M. Houweling and M. Bussière, unpublished observations). Therefore, we reasoned that ceramide might inhibit axonal growth by inhibiting the axonal synthesis of PC. Ceramide, however, did not inhibit PC synthesis within axons (Fig. 6.4).

Both the growth-inhibitory effect of ceramide (Chapter 5 and Fig. 6.2) and the growth-promoting effect of NGF (3) on axonal growth are localized to the axon terminals or growth cones of rat sympathetic neurons. It is possible, therefore, that ceramide might impair axonal growth by interfering with NGF signal transduction which activates the cellular machinery necessary for axonal growth. As mentioned previously, most of the effects of NGF on neuronal growth and survival are attributed to the binding of NGF to trkA, a receptor tyrosine kinase (3-5). Evidence suggests that the initial steps in the mechanism of NGF action are: the association of NGF with trkA, trkA tyrosine autophosphorylation and the tyrosine phosphorylation of a number of adaptor and effector proteins (4, 5). Treatment with exogenous bacterial SMase and cell permeant analogs of ceramide has recently been shown to attenuate the effects of insulin on cells by inhibiting insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation (30). Similar results were obtained when the effect of ceramide on NGF-induced protein tyrosine phosphorylation was examined (Fig. 6.5B). Maximal protein tyrosine phosphorylation was observed after 1 h of treatment with 20 ng/ml NGF and was sustained for at least 2 h. Previous studies have shown that protein tyrosine phosphorylation in response to NGF is sustained upwards of 24 h in cultured

rat sympathetic neurons (6). In contrast, maximal protein tyrosine phosphorylation induced by 20 ng/ml NGF in the presence of 25 μM C_6-ceramide was observed within 30 min of treatment after which tyrosine phosphorylation diminished (Fig. 6.5B). Ceramide treatment itself has also been shown to induce the tyrosine phosphorylation of proteins in cells (31-33). No increased protein tyrosine phosphorylation was detected upon treatment of sympathetic neurons with ceramide (Fig. 6.6), however. Previous studies have demonstrated that NGF-trkA signaling involved in regulating axonal growth is localized to distal axons of rat sympathetic neurons (3, 6). The results described in this Chapter suggest that ceramide might inhibit axonal growth by attenuating or interfering with NGF-induced signal transduction within distal axons.

Studies in this Chapter have defined further the process of axonal growth inhibition by ceramide. This work is the basis for future studies aimed at identifying the signaling pathways and mechanisms by which ceramide inhibits axonal growth (see Chapter 7).

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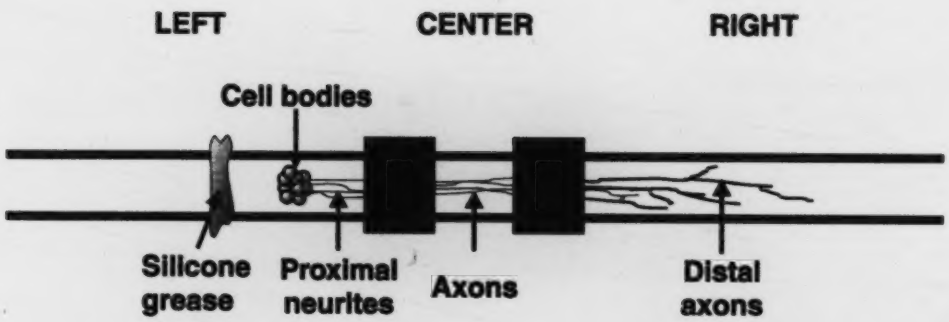


Fig. 6.1 **Schematic diagram of a single track of a left-plated compartmented culture**

An enlargement of a single track of a left-plated compartmented culture is illustrated. Cell bodies isolated from enzymatically and mechanically dissociated ganglia are plated in a pocket in the left compartment of dishes formed by carefully applied silicone grease. After 2 weeks of growth, the left side compartment contains cell bodies and proximal neurites, the center compartment contains axons and the right side compartment contains axons and axon terminals.

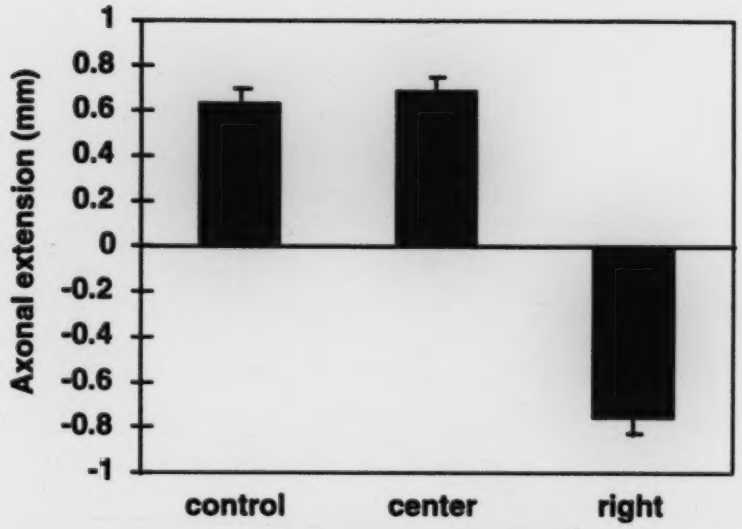


Fig. 6.2 **The inhibitory effect of ceramide on axonal growth is localized to axon terminals**

Left-plated compartmented cultures of neurons were grown for 13 days and then neuritotomized and allowed to regenerate for 3 days. Axonal extension was measured and then cultures were either left untreated, treated with 10 μM C_6-ceramide in the right side compartment or treated with 30 μM C_6-ceramide in the center compartment. Axonal extension was again measured 24 h later. Results are the mean axonal elongation in 24 h ± S.E. of measurements from 60 to 80 tracks for each treatment. The experiment was repeated once with similar results.

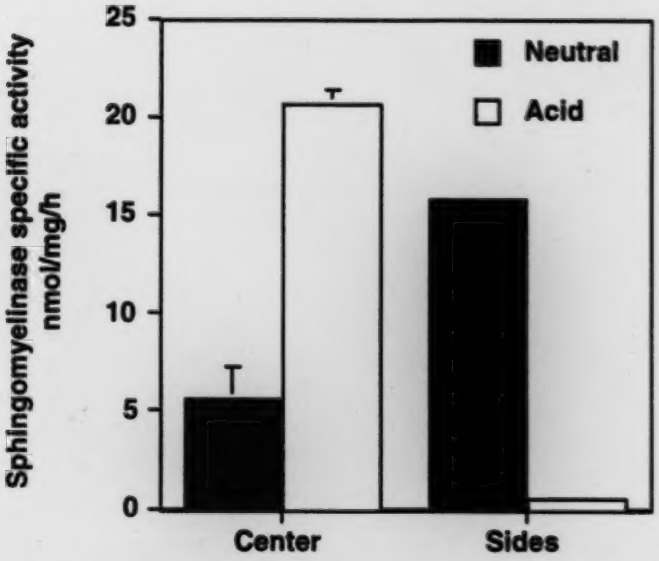


Fig. 6.3 **Acid sphingomyelinase localizes to cell bodies and neutral sphingomyelinase localizes to axons and axon terminals**

Neurons were cultured for 13 days in center-plated compartmented culture dishes. Cultures were washed and the cellular material from the center or side compartments was harvested separately and assayed for neutral or acid SMase activity. Shown is the mean specific enzyme activity ± S.D. of 4 replicate determinations. The experiment was repeated twice with similar results.

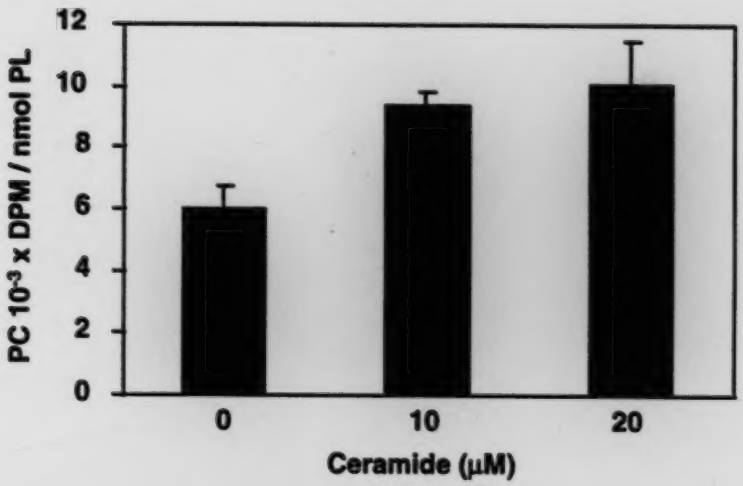
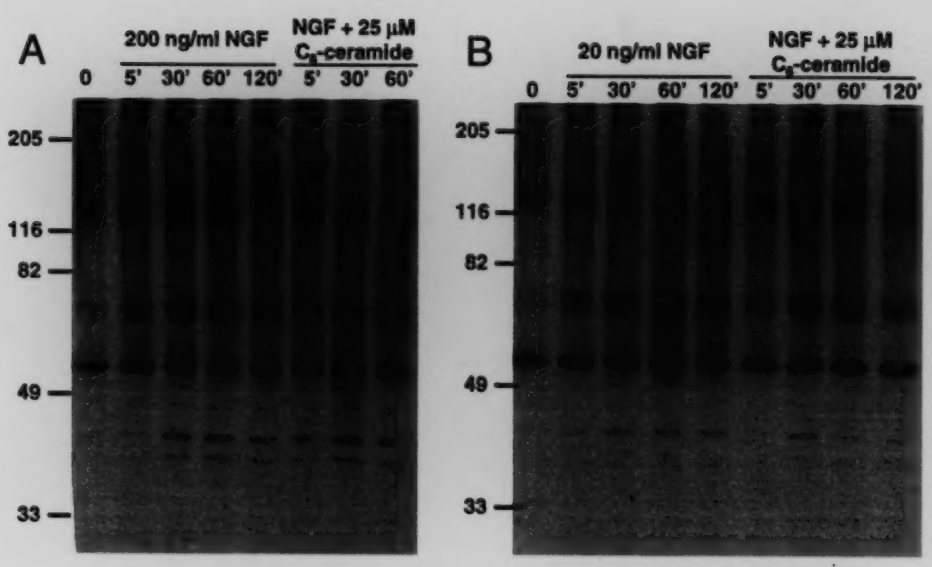


Fig. 6.4 **Ceramide does not inhibit axonal PC synthesis**

The side compartments of 13-day old center-plated compartmented cultures of neurons were given [^{3}H]choline (10 μCi/ml) for 6 h in the absence or presence of 10 or 20 μM C_6-ceramide. Cultures were washed three times with ice-cold Tris-buffered saline and the cellular material in the side compartments was harvested, the lipids extracted, separated by thin-layer chromatography and the incorporation of [^{3}H]choline into PC was determined. Each value represents the mean ± S.D. of 3 replicate determinations. The experiment was repeated once with similar results.

Fig. 6.5 **Ceramide alters the time course of NGF-induced protein tyrosine phosphorylation**

Center-plated compartmented cultures of neurons grown for 10 days in medium containing 100 ng/ml NGF in the side compartments were washed twice with NGF-free medium and incubated for 3 days with medium containing 20 ng/ml NGF. On the day of the experiment, cultures were washed twice with NGF-free medium and incubated for 6 h in the absence of growth factors or serum. At this time, the side compartments of cultures were given medium containing either 200 ng/ml NGF with or without 25 μM C_6-ceramide (panel A) or 20 ng/ml NGF with or without 25 μM C_6-ceramide (panel B), as indicated. Extracts of the cellular material from the side compartments were prepared at various times after treatment. Equal amounts of protein were run on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose, Western blotted with antiphosphotyrosine antibody and detected using enhanced chemiluminescence. The experiment in panel A was repeated twice and the experiment in panel B was repeated once with similar results. Prestained molecular mass standards (Bio-Rad) include: myosin (205 kDa), β-galactosidase (116 kDa), bovine serum albumin (82 kDa), ovalbumin (49 kDa), carbonic anhydrase (33 kDa).


A
200 ng/ml NGF
NGF + 25 μM
C_2-ceramide
0
5'
30'
60'
120'
5'
30'
60'
205
116
82
49
33
B
20 ng/ml NGF
NGF + 25 μM
C_2-ceramide
0
5'
30'
60'
120'
5'
30'
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120'
205
116
82
49
33

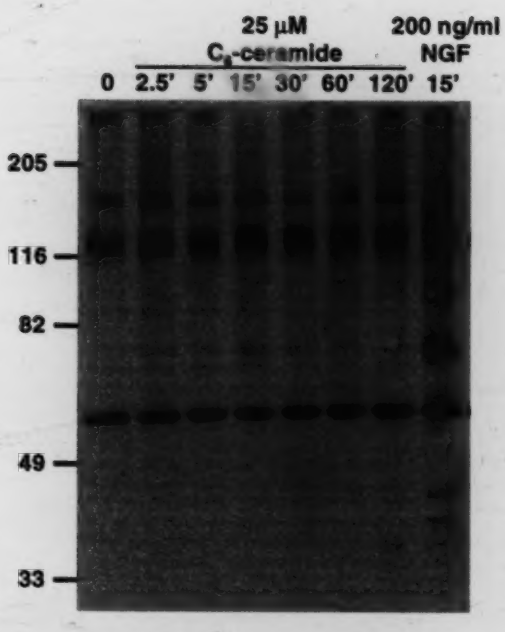

Fig. 6.6 **Ceramide does not induce the tyrosine phosphorylation of proteins**

This experiment was conducted essentially as described in Fig. 6.5 except that cultures were either treated with 25 μM C_6-ceramide alone or with 200 ng/ml NGF alone. The experiment was repeated three times. Although, an increase in the tyrosine phosphorylation of the 140 kDa band is apparent after 60 and 120 min of C_6-ceramide treatment in this Western blot, this result was not obtained in replicate experiments.

CHAPTER 7

SUMMARY, CONCLUDING REMARKS AND FUTURE DIRECTIONS

As stated in Chapter 1, choline is utilized for the synthesis of membrane PC in all eukaryotic cells. In cholinergic neurons, choline is also utilized for synthesis of the neurotransmitter Ach. The first part of this thesis, Chapters 1, 2 and 3, deals with the regulation of choline metabolism in cholinergic neurons and the mechanisms involved in the distribution of choline between PC and Ach synthesis.

In Chapter 2, a cell culture system was established to investigate the metabolic changes that occur upon cholinergic induction. Sympathetic neurons, isolated from the superior cervical ganglia of rats, are adrenergic and therefore synthesize and secrete the neurotransmitter norepinephrine (1). Prolonged treatment of these neurons in culture with RA or leukemia inhibitory factor causes a switch in neurotransmitter phenotype (2, 3). The neurons stop producing norepinephrine and begin to produce acetylcholine as their neurotransmitter, a process called cholinergic differentiation. Studies in Chapter 2 examined the changes in choline metabolism occurring in sympathetic neurons undergoing cholinergic differentiation. Examination of the specific enzyme activity of ChAT and CK during differentiation induced by RA or leukemia inhibitory factor suggests that these enzymes might be reciprocally regulated. Induction of cholinergicity resulted in an increase in ChAT activity with a concomitant decrease in CK specific activity, thus effectively diverting a portion of choline destined for PC synthesis into Ach synthesis. This decrease in CK activity, however, did not compromise overall PC synthesis. The rate limiting step of PC synthesis in cultured rat sympathetic neurons is the conversion of phosphocholine to

CDP-choline catalyzed by the enzyme CT (4, 5). Even upon cholinergic differentiation, phosphocholine must be supplied in excess to CT since a decrease in PC synthesis was not observed.

Two characteristics have been used to identify cholinergic neurons: ChAT activity and HACT (6-10). Extracellular choline taken up by HACT is thought to be the immediate source of choline for Ach synthesis (6-10). Increased Ach synthesis can be caused either by an increase in ChAT activity or by an increased rate of choline transport by HACT (9, 10). In Chapter 2, HACT was shown to be present in cultures of rat sympathetic neurons and was unaffected by RA-treatment. NGF, required for the growth and survival of sympathetic neurons in culture, has been demonstrated to increase both ChAT and HACT in certain populations of neurons (11-13), and might account for the presence of HACT in cultures of sympathetic neurons. As has been demonstrated to occur in other cell types and in synaptosomal preparations (14-18), choline taken up by HACT was utilized both for Ach and PC synthesis in sympathetic neurons.

An important aspect of the regulation of choline metabolism in cholinergic neurons is the topography of Ach and PC synthesis. PC is synthesized both in the cell bodies and axons of neurons (4, 5). Axonal synthesis of PC has been shown to be critical for the generation of new membranes required for axonal growth and regeneration (19). Evidence suggests that Ach is synthesized predominantly in the axon terminals of

neurons (6-10). Subcellular fractionation and denervation studies suggest that both ChAT and HACT are enriched in the axon terminals of cholinergic neurons (reviewed in 6-10), which explains the restricted localization of Ach synthesis. In Chapter 3, compartmented cultures of neurons were utilized to examine the topography of Ach synthesis and HACT. Evidence was provided which demonstrates that Ach is synthesized exclusively in the distal axons of RA-treated sympathetic neurons. Furthermore, only choline supplied directly to distal axons was incorporated into Ach. Choline taken up by cell bodies and proximal neurites and transported to the distal axons was incorporated into PC but not into Ach. In accordance with previous studies (6-10) and with the restricted localization of Ach synthesis, HACT was found to be present only in the distal axons of sympathetic neurons. The sum of data from Chapter 2 and 3 are in agreement with a study by Suidan and Tolkovsky (20) which concluded that sympathetic neurons are poised to allocate choline symmetrically between PC and Ach synthesis at their growing axon endings.

Future investigations could involve examining changes in the specific enzyme activity of CK during cholinergic differentiation in compartmented cultures of sympathetic neurons. Studies in Chapter 2, demonstrated that CK specific activity decreased upon cholinergic induction. It is possible that the decrease in overall CK activity is due to a local decrease in CK activity within distal axons, but not within cell bodies. Several isoforms of CK have been detected in brain and liver (21, 22). Little is known, however, about the cellular function of these isoforms of CK. A decrease in the

activity of a specific isoform of CK could account for the decrease in CK enzyme activity observed upon RA-treatment.

The second part of this Thesis, Chapters 4, 5 and 6, examines the role of sphingolipids and GSLs in neurons, specifically in the process of axonal growth. As mentioned in Chapter 4, which summarizes the background of the second half of this Thesis, sphingolipids are abundant constituents of neuronal membranes and many investigations have focused on determining the role of complex sphingolipids, such as GSLs and gangliosides, in neuronal cell function. The enrichment of gangliosides in the plasma membrane of neural cells induces neuritogenesis (23-26). Furthermore, antibodies raised against gangliosides inhibit axonal outgrowth from neural cells and tissues slices *in vitro* (27, 28). These studies strongly suggest that gangliosides play a key role in axonal growth. In Chapter 5, the potential role of GSLs and gangliosides in axonal growth was examined by reducing, instead of augmenting, endogenous GSL levels using two inhibitors of the synthesis of GSLs, FB_1 and PPMP. Although both of these compounds were shown to inhibit the synthesis of GSLs in cultured rat sympathetic neurons, only PPMP, but not FB_1, inhibited axonal growth and PPMP inhibited axonal growth only when applied to distal axons. Several lines of evidence from this study implied that the distinct effects of FB_1 and PPMP on axonal growth were related to their differential effects on ceramide metabolism. PPMP induced an accumulation, whereas FB_1 caused a reduction, of [^{3}H]palmitate-labeled ceramide.

Moreover, pretreatment of neurons with FB_1 prevented the inhibitory effect of PPMP on axonal growth and exogenously-added cell-permeant ceramides mimicked the effect of PPMP on axonal growth. Contrary to previous studies (23-28), these experiments suggest that newly synthesized gangliosides are not essential for axonal growth. The results suggest, however, that the lipid second messenger ceramide acts in distal axons, but not cell bodies, as a negative regulator of axonal growth.

One issue that arises from the results obtained in Chapter 5, is the subcellular location of glucosylceramide synthesis in neurons. As described above, several lines of evidence demonstrate that the effect of PPMP on axonal growth is mediated by an increase in ceramide within distal axons treated with PPMP. Since PPMP is an inhibitor of the enzyme glucosylceramide synthase, this data suggests that glucosylceramide is synthesized in distal axons. As described in Chapter 4, the initial steps of GSL synthesis leading to the production of ceramide occur on the cytosolic surface of the endoplasmic reticulum (29, 30). The location of glucosylceramide synthesis has been the subject of many investigations. Evidence suggests that glucosylceramide is synthesized on the cytosolic surface of Golgi membranes and in a pre-Golgi-compartment (31-35). The subsequent glycosylation steps generating more complex GSLs and gangliosides are thought to occur within the Golgi complex (36, 37). In neurons, the Golgi apparatus and rough endoplasmic reticulum are restricted to the cell body, the site of protein synthesis. Smooth endoplasmic reticulum, however, is present both within cell bodies and in the dendrites and axons of neurons, where it

is often referred to as axoplasmic reticulum (38-40). Consistent with the presence of endoplasmic reticulum membranes in axons, axons are capable of synthesizing membrane lipids, including phospholipids and sphingomyelin (4, 5). The available data suggests that the synthesis of glucosylceramide occurs in axons probably on or within the axoplasmic reticulum. A goal of future investigations could be to demonstrate *de novo* glucosylceramide synthesis within axons.

An increase in intracellular ceramide can be produced by the breakdown of membrane SM, and perhaps GSLs (see Chapter 4 Section 4.4.1 and 4.4.2), as a biological response to extracellular agents or insults. SM turnover as a signaling event has not been demonstrated in nervous tissue. A goal of future investigations could be to determine if the neurotrophins, and other agents such as TNF-α, can induce SM hydrolysis and ceramide production in primary neurons and consequently affect neuronal cell function. The neurotrophins induce SM hydrolysis in cultured cells expressing the p75 neurotrophin receptor ($p75^{NTR}$) (41, 42). The role of $p75^{NTR}$ in neurotrophin function has not been clearly elucidated but recent studies have demonstrated that $p75^{NTR}$ can initiate cell death signals (43-46). Additional studies are required to determine if ceramide generation is required for the induction of apoptosis via $p75^{NTR}$ and also to determine if activation of ceramide signaling via $p75^{NTR}$ is involved in the regulation of axonal growth.

Several transmembrane and secreted proteins have been identified, including myelin-associated glycoprotein and the semaphorins, which inhibit axonal growth by causing growth cone collapse (47-50). These proteins are thought to play a key role in neuronal pathfinding during development by acting as repulsive axonal guidance cues (49-52). The signaling mechanisms activated by these proteins which mediate growth cone collapse and axonal growth inhibition have not been elucidated but could involve the local generation of ceramide within axon terminals.

Both acid and neutral SMase have been proposed to function in the generation of ceramide as a signaling event (51, 52). In Chapter 6, acid SMase activity was shown to be localized to cell bodies and Mg^{2+}-dependent neutral SMase activity was found to be enriched in axons and axon terminals of rat sympathetic neurons. In addition, the effect of ceramide on axonal growth was shown to be restricted to axon terminals and axonal transport of ceramide was found to be inefficient. Ceramide involved in the regulation of axonal growth must, therefore, be generated locally, within or near axon terminals. Since Mg^{2+}-dependent neutral SMase activity is enriched in this cellular location, and acid SMase activity is absent, activation of Mg^{2+}-dependent neutral SMase in sympathetic neurons could generate ceramide and inhibit axonal growth.

The mechanism of ceramide-mediated inhibition of axonal growth remains to be elucidated and this question is certain to be the subject of many future investigations. It is clear from studies in Chapter 5 and 6 of this thesis, that ceramide inhibits axonal

elongation by affecting growth mechanisms localized to distal axons, possibly in axon terminals or growth cones. The mechanisms involved in the stimulation of axonal growth in response to NGF are also localized to distal axons. Ceramide treatment appears to attenuate NGF-activated protein tyrosine phosphorylation. Although the pattern of NGF-induced protein tyrosine phosphorylation within distal axons was unaffected by ceramide treatment, phosphorylation was not as prolonged or pronounced in the presence of ceramide (Chapter 6 Fig. 6.5B). Thus, one possible mechanism of ceramide-mediated inhibition of axonal growth is the attenuation of or interference with NGF signaling in distal axons. Future experiments could involve reexamining the effect of ceramide on NGF-induced protein tyrosine phosphorylation over a greater time course and examination of the time course of NGF-induced tyrosine phosphorylation of trkA specifically (and other proteins) in the absence and presence of ceramide by immunoprecipitation followed by anti-phosphotyrosine immunoblotting. These experiments would provide further support for an effect of ceramide on NGF-signaling.

As described in Chapter 4, several direct molecular targets for ceramide have been identified which are involved in mediating the effects of ceramide on cells including: ceramide-activated protein phosphatase (CAPP), ceramide-activated protein kinase (CAPK) and protein kinase C ζ. Although CAPP activity has been detected in rat brain homogenates (53) and therefore might have a role in neuronal cell function, several lines of evidence suggest that CAPP is not involved in ceramide-mediated

inhibition of axonal growth. CAPP is a putative PP2A protein phosphatase which is activated by ceramide and inhibited by okadaic acid *in vitro* (54-57). Treatment of cells with low concentrations of okadaic acid prevents some of the cellular responses to ceramide (54, 57). In studies investigating the effect of okadaic acid on axonal growth, okadaic acid was found to inhibit axonal elongation even at low nanomolar concentrations (1-10 nM) (R. Vance and R.B. Campenot, unpublished results). If CAPP mediates the inhibitory effect of ceramide on axonal growth, an inhibitory effect of okadaic acid on axonal growth would not be predicted. Furthermore, despite extensive efforts to detect CAPP activity *in vitro* in homogenates of either rat sympathetic neurons or rat brain, or using purified PP2A from *Saccharomyces pombe*, we were unable to detect any stimulation of PP2A activity due to ceramide treatment (M. Bussiere, J. Dawson, C. Holmes and Vance, D.E.). The involvement of CAPK and protein kinase C ζ in ceramide-mediated inhibition of axonal growth remains to be evaluated.

The stress-activated protein kinase (SAPK) pathway and the p38 kinase pathway are two major signaling pathways activated in response to stress and lead to differentiation, growth arrest or apoptosis (58-59). These two pathways warrant further investigation as potential pathways involved in ceramide-mediated inhibition of axonal growth. Ceramide has been shown to activate the SAPK cascade in cells (60-62). Either one or both of these stress-activated pathways might be involved in the inhibition of axonal growth by ceramide.

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